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13. ABSTRACT *(Maximum 200 Words)*

Many forms of metastatic carcinoma are characterized by the lack of MHC I expression on their cell surface due to the down-regulation of transporters associated with antigen processing. Increasing expression of TAP in TAP deficient small cell lung cancer and melanoma induces a protective, anti-tumor immune response. Clinical studies show that a significant percentage of high grade breast carcinomas show MHC I loss and TAP deficiency. We hypothesize that increasing TAP expression in breast cancers will increase the cancer's immunogenicity. To test this hypothesis we screened breast cancer cell lines for deficiencies in antigen presentation with the aim of developing a tumor model in mice capable of responding to vaccinia-TAP expression vectors. The breast tumor cell lines screened expressed MHCI on the cell surface and expressed TAP. These cell lines formed tumors and were resistant to vaccinia-TAP treatment. The strain of mouse (BALBc) syngenic to the tumor cell lines, however, is not a good candidate to test therapies dependent on cytotoxic responses. Instead we are searching for breast cancer cell lines syngeneic with C57 black strains of mice which are able to generate a protective, Th1 immune response.

14. SUBJECT TERMS
breast cancer, MHCI, TAP

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Table of Contents

Introduction

Many cancers are not detected by the immune system because they lack the expression of MHC class I (MHC I) on the cell surface. MHC I loss is often due to defects in the antigen presentation pathway that include absence of proteins such as LMP-2, LMP-7, TAP-1, TAP-2, Tapasin, and beta2-microglobulin. Examination of resected tumors from cancer patients reveals a high percentage of carcinomas lacking full TAP expression. Despite the multiple defects, restoration of TAPs (transporters associated with antigen processing) alone in carcinoma cell lines can restore presentation of tumor antigens at the cell surface and allow recognition by specific cytotoxic T lymphocytes (CTL). For example, the small cell lung cancer cell line, CMT 64 does not express MHC I on the cell surface and is devoid of TAP 1 and TAP 2 expression. Introduction of TAP-1 into CMT 64 cells restores MHC I expression on the cell. Introduction of TAP positive CMT 64 cells into syngeneic mice leads to the generation of protective, specific, CTL responses against the malignant cells. In contrast, introduction of TAP negative CMT 64 cells into syngeneic mice leads to 100% mortality within 4 weeks (appendix I). These findings have been extended to the melanoma cell line B16F10. This cell line is also deficient in TAPs and does not express MHC I on the cell surface. Syngeneic mice bearing TAP negative B16F10 tumors were treated with a vaccinia based expression vector carrying the TAP gene (VV hTAP). Tumor growth was significantly retarded in treated mice compared to sham treated mice (data not published, Appendix II). These two studies suggest that restoring expression of TAPs in tumors using an expression vector could be used as a therapeutic intervention. We aim to extend our findings on small cell lung cancer and melanoma to breast cancer. The objectives of this study are to 1) characterized the phenotype of mouse breast cancer cell lines for MHC I and TAP expression, 2) examine if increasing TAP expression in breast cancer tumors *in vivo* by treatment with VV-hTAP can generate protective immune responses and retard tumor growth.

Body

Specific Aim 1: Characterization of phenotype of mouse breast cancer cell lines for MHC I and TAP expression.

The following mouse breast cancer cell lines were obtained from the ATCC (Bethesda , Maryland) (Table 1).

Table 1. Mice breast tumor cell lines characterized for MHC I, TAP expression and tumorgenicity.

Cell Line	Mouse Strain	Source
CL-S1	BALBc	ATCC
JC	BALBc	ATCC
4T1	BALBc	ATCC

We first analyzed expression of the MHC class I molecules on tumor cells by FACS assay (see figure 1). Using antibodies, HB159 and 3.4.12.5 against Kd and Dd, repectively, we found the cell line, CL-S1, does not express MHC class I while JC and 4T1, both express high level of K^d and D^d molecules on the cell surface. Interferon-gamma (IFN-γ stimulates MHC expression) treatment enhances MHC class I expression only in 4T1 cells but not in CL-S1 and JC cells. In accordance with MHC I expression, the three cell lines display different TAP expression. Western blot analysis shows that CL-S1 cells express none or very little TAP1 and TAP2 proteins while JC and 4T1 cells express higher amount TAP1 and TAP2 proteins (figure 2). These experiments indicate that JC and 4T1 breast tumor cell lines express major components of MHC class I antigen presentation machinery and are not deficient in TAP protein expression. We tested the tumorgenicity of the 3 cell lines by introducing various amounts of viable cancer cells into syngeneic BALBc mice (Table 2).

Table 2. Tumorgenicity of breast cancer cell lines.

Cell Line	Cancer load injected	First Appearance of Tumor
CL-S1	$5x10^5$	No tumor growth
JC	$5x10^5$	15 days
4T1	$5x10^5$	8 days

Specific Aim 2: To examine if increasing TAP expression in breast cancer tumors *in vivo* by treatment with VV-hTAP can generate protective immune responses and retard tumor growth.

We have successfully established the use of VV-TAP as a proof of principle in the treatment of TAP deficient small cell lung cancer and melanoma (see appendix I and II) These experiments were performed using TAP-negative cell lines with the rationale that delivering the TAP gene into some TAP-negative cancer can increase the capacity of antigen presentation of tumor cells and drive a Th1 immune response against the tumor.

Originally, we planned to extend these studies to TAP negative breast cancer cell lines based on clinical studies that demonstrate an association between down-regulation of TAPs, loss of MHC I, and tumor progression. High grade primary breast carcinoma lesions have the highest prevalence of MHC I loss and TAP down-regulation (Vitale *et al.,* 1998). The breast cancer cell lines screened by us, however, were not deficient in TAPs or MHC I. Increasing TAP expression in cancer cell lines that already express TAPs may still increase immunogenicity of the cancer. This is based on the fact that tumor antigens are self-antigens and

are weak immunogens, but increased immune stimulation could break tolerance against the tumor. Such stimulations include pulsing dendritic cells with tumor lysates and tumor antigens, and/or immunizing with irradiated cancer cells pulsed with tumor-antigen peptides.

Introducing TAPs into cancers cells with a vaccinia virus in order to increase TAP expression should change the tumor antigen profile on the cell surface both qualitatively and quantitatively. This may provide an increased stimulus for the immune system allowing a break in tolerance against the tumor. In addition if VV-TAP infects antigen-presenting cells the resultant over expression of TAP will increase the antigen presentation capacity. This will lead to higher T cell generation (manuscript in preparation, Appendix III).

We tested the hypothesis that delivering VV-TAP to TAP positive tumors will enhance TAP expression leading to an increased anti-tumor response. Mice (BALBc strain) were given $5x10^5$ cancer cells (JC and 4T1) subcutaneous. One day after the introduction of the tumor cells mice received $1x10^6$ plaque forming units (pfu) of vaccinia-TAP1 or vaccinia-TAP1+2, injected in the region of the growing tumor. This was followed by another dose, equal to the first, 7 days later. Negative controls consisted of sham treatments with PBS. Another group received vaccinia delivering the plasmid pJS-5 to control for the effect of the vector. Twenty days after the introduction of the tumors, mice were killed and the tumors resected and weighed.

No difference in tumor mass was detected between the treatment and control groups (figures 3 & 4). These preliminary experiments suggest that augmenting TAP expression in TAP positive tumors is not sufficient to break tolerance and generate anti-tumor immune responses. The results may be in part reflective of the syngeneic tumor model available to us. One possible factor that needs to be explored is the type of immune response generated by BALBc mice. For instance the immune response to virus infection can differ depending on the gender of BALBc mice. The immune response to coxsackie virus in female BALBc mice results in a Th2 response that may be potentially inhibitory to anti-tumor CTL responses. In male BALBc the immune response to the same virus is a Th1 type response which is responsible for anti-tumor activity (Huber *et al.*, 1994). In future studies we will continue to characterize and optimize our tumor models to assess the utility of TAP as an immunotherapy for breast cancer.

References:

Huber *et al.* J. Virol. 1994, Aug; (68(8):5126-32
Vitale *et al., Cancer Research* 1998 Feb 15; 58(4): 737-42

Key Research Accomplishments:

- Characterized MHC I expression on three breast cancer cell lines (CL-S1, JC, 4T1).
- Measured induction of MHC I expression on three breast cancer cell lines in response to interferon-gamma treatment (CL-S1, JC, 4T1).
- Characterized the expression of TAP in three breast cancer cell lines (CL-S1, JC, 4T1) by Western Blot analysis.
- Determined the tumorgenicity of three breast cancer cell lines (CL-S1, JC, 4T1).
- Measured the ability of vaccinia-TAP treatment to control the growth of two breast cancer cells in mice (JC and 4T1).

Reportable Outcomes:

Manuscripts

N/A

Abstracts

Potential Significance of TAP1-Based Viral Delivery System on Tumor-Immunotherapy

Qian-Jin Zhang*, Susan Chen*, Xiao-Lin Li, Andrew P. Jeffries, Rayshad S. Gopaul, Kyang-Bok Choi and Wilf. A. Jefferies
The Biotechnology Laboratory, Biomedical Research Centre, and The Departments of Medical Genetics, Microbiology and Immunology, and
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*These authors contributed equally to this work

Tumor cells may escape the T cell-based immunosurveillance through various mechanisms, including loss or down-regulation of TAP expression and/or induction of cytokines such as IL-10 and IL-4. The loss or down-regulation of TAP expression prevents presentation of the tumor-associated antigen (TAA) on the surface of tumor cells and therefore they avoid to be recognized by specific T cells. On the other hand, high expression of IL-10 and/or IL-4 in tumors inhibits generation of tumor specific T cells. The mouse melanoma cell line, B16F10, is one example of tumor cells that down-regulates antigen presentation machinery (including TAP) and induces IL-10 production. Here we have used this tumor model to test the hypothesis that TAP down-regulation in tumors allows immuno-subversion of this effector mechanism. The TAP transfectants was established to examine the role of TAP in restoring antigen presentation, immune recognition and effects on malignancy of the TAP-deficient B16F10. In order to address the potential of providing exogenous TAP in cancer therapies, a vaccinia virus construct containing the TAP1 gene was used to examine whether VV-TAP1

could enhance tumor specific T cell generation and reduce tumor-burden in mice. The results demonstrate that the inclusion of TAP-based viral delivery system in cancer therapies should be considered, as it is likely to provide a general method for increasing immune responses against tumors regardless of the antigenic complement of the tumor or the MHC haplotypes of the host.

Presentations

Qian-Jin Zhang*, Susan Chen*, Xiao-Lin Li, Andrew P. Jeffries, Rayshad S. Gopaul, Kyang-Bok Choi and *Wilfred. A. Jefferies*. Potential Significance of TAP1-Based Viral Delivery System on Tumor-Immunotherapy, CANVAC meeting, Montreal, Quebec. March 2002.

Patents and licenses applied for and/or issued
N/A

Degrees obtained that were supported by this award:
Although no degrees were obtained by this one year award, graduate students were supported through this award as their work was directly related to the project.

Development of cell lines, tissue or serum repositories
Transfected rat TAP cell line

Animal Models
C57 b black mice injected with B-16 cell line.
Balbc mice injected with the CL-S1 cell line, the JC cell line, and the 4T1 cell line.

Funding applied for based on work supported by this award
N/A

Employment or research opportunities applied for and /or received based on experience/training supported by this award
N/A

Personnel that received a salary from the award:

Dr. Maki Ujiie: Postdoctoral fellow
Mr. Jason Grant: Graduate student
Fonny Fong: animal technician
Shahriar Tabrizi: Animal technician
Kiong Bok Choi: Molecular biology technician

Conclusions:

MHC I and TAP expression characterize the phenotype of the mouse breast cancer cell lines and these were shown to form tumors in mice. Treatment of these tumors with vaccine-TAP was not able to retard their growth. This is in contrast to small cell lung cancer and melanoma that we have studied previously. We hypothesize that female BALBc mice are unable to generate the Th1 $CD4^+$ helper response that is required for anti-tumor immune activity. Further studies are required to refine the tumor models used for the evaluation of TAP as an immuno-therapy for the treatment of breast cancer. Future studies should include mouse strains that are capable of generating Th1 type helper responses as well as the identification of appropriate syngeneic cancer cell line.

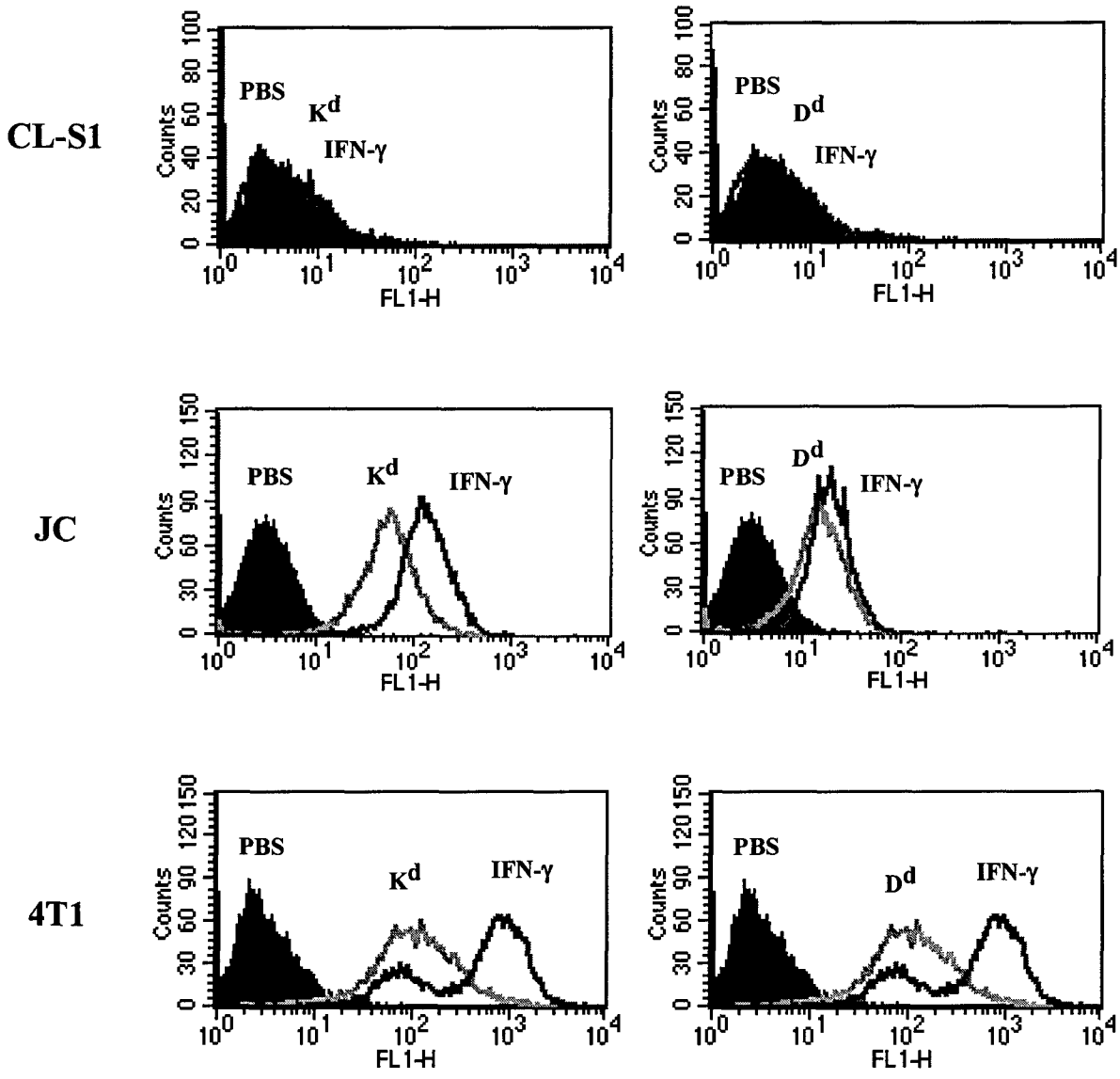

Figure 1. Detection of surface MHC class I expression in breast tumor cell lines. Surface K^d and D^d expression in CL-S1, JC and 4T1 cells were detected by FACS assay using monoclone antibodies agaist

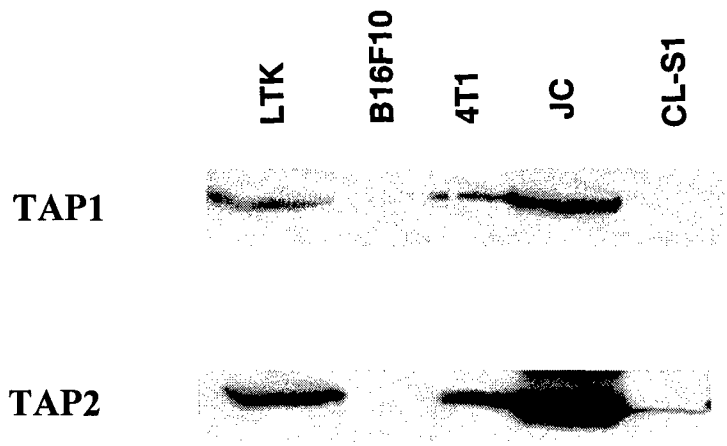

Figure 2. Detection of endogenouse TAP expression on breast tumor cell lines. Western blot analysis was performed to detect TAP expression on 4T1, JC and CL-S1 cells by using rabbit antisera against mouse TAP1 or mouse TAP2. The LTK and B16F10 cell lines used here as positive or negative controls.

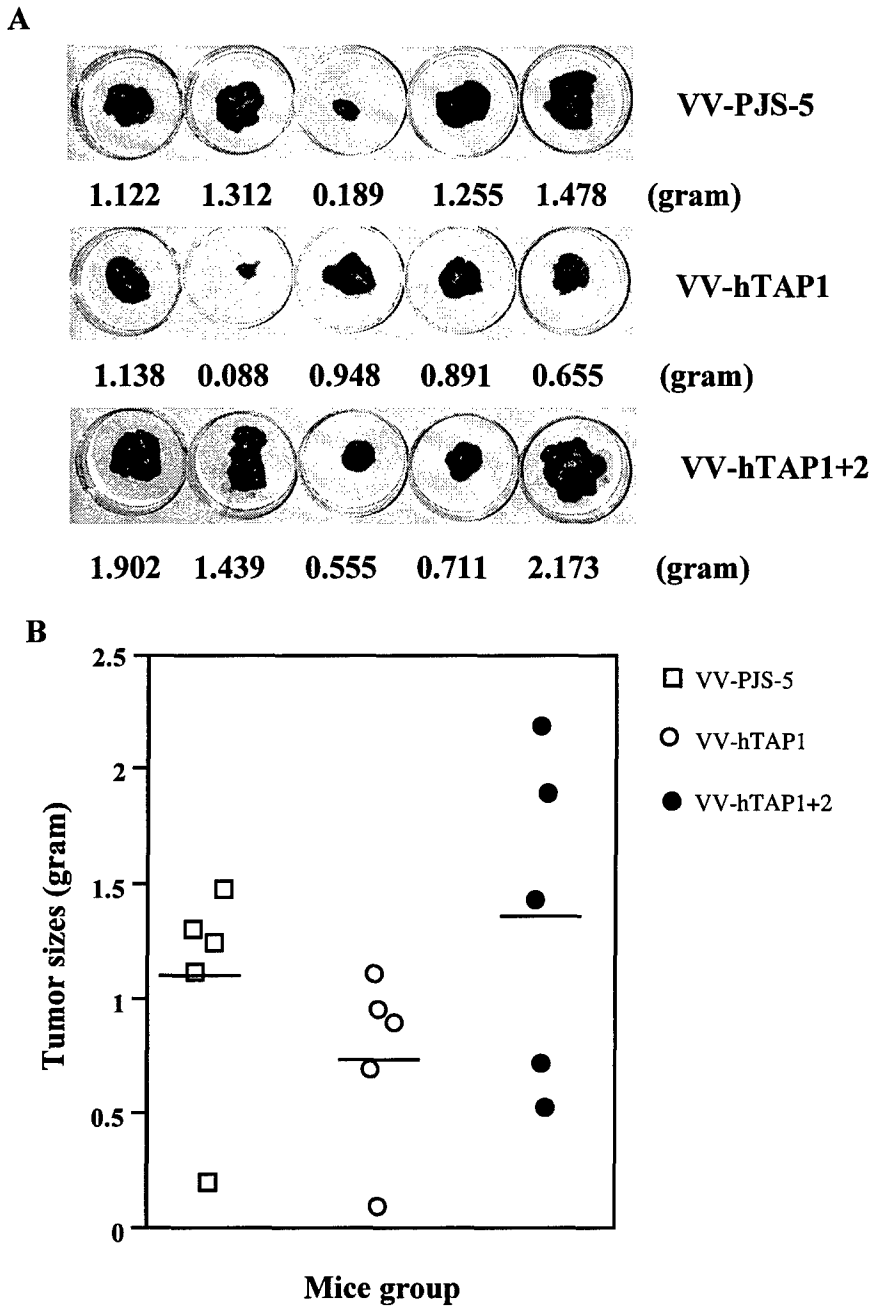

Figure 3. TAP-vaccinia virus treatment cannot significantly inhibit the growth of TAP-positive tumor, JC, in BALBc mice. Mice were subcutaneously inoculated with $5X10^5$ JC cells per mouse and in next day they were injected $1x10^6$ PFU vaccinia virus carrying with or without human TAP1 or TAP 1+2 genes into same loci. Each group contains five mice. This treatment were boosted at day seven of tumor inoculation. After thirty-five days, all mice were sacrificed and the tumor sizes were measured. (A) tumor sizes for each group are shown. (B) statistical analysis of t-test shows that in VV-hTAP1 or VV-hTAP1+2 treated group mice tumor growth cannot be inhibited by body immune system comparing to VV-PJS-5 (vactor alone) group mice, one-side P value > 0.05.

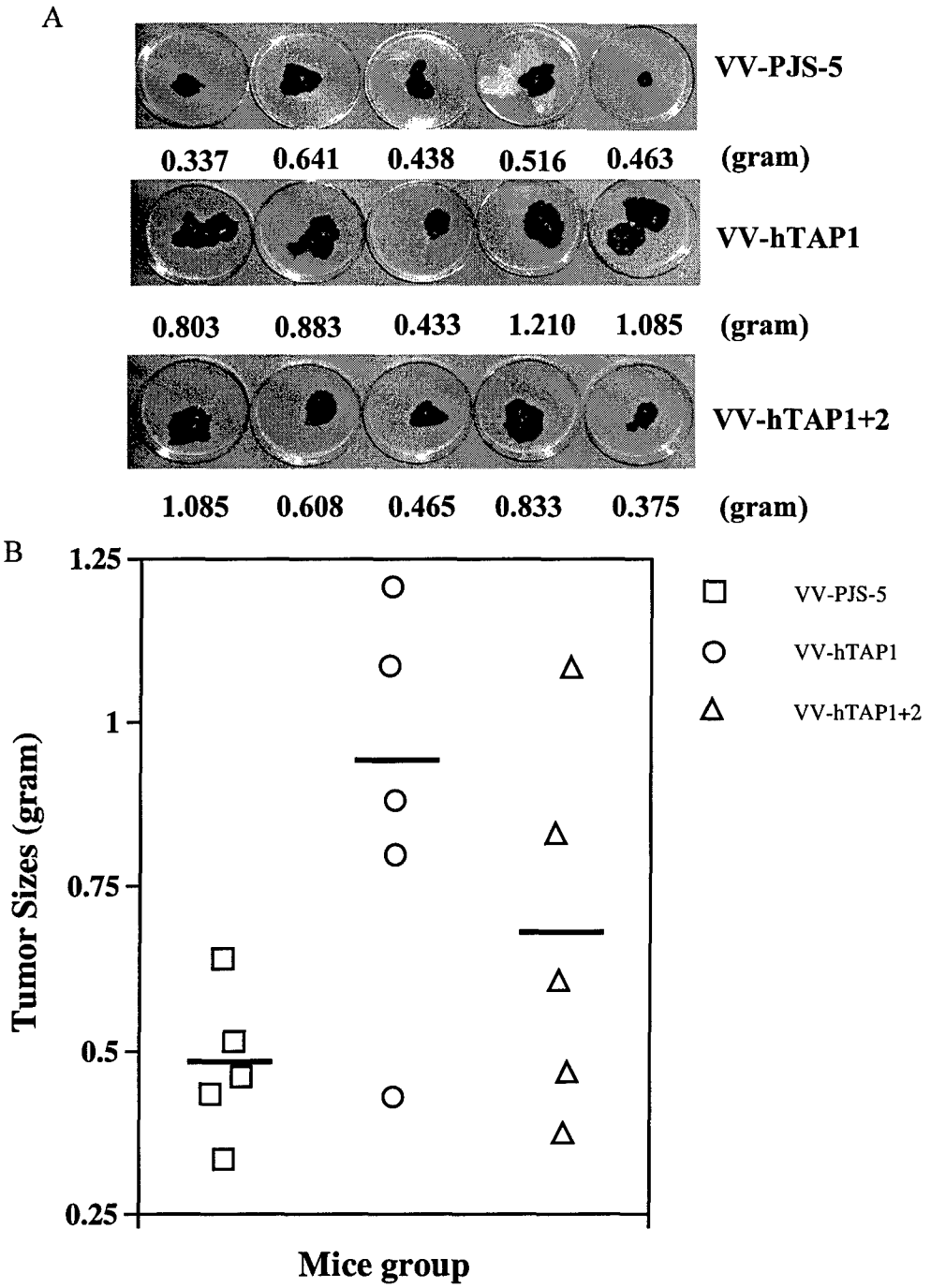

Figure 4. TAP-vaccinia virus treatment cannot significantly inhibit the growth of TAP-positive tumor, 4T1, in BALBc mice. Mice were subcutaneously inoculated with $5X10^5$ 4T1 cells per mouse and in next day they were injected $1x10^6$ PFU vaccinia virus carrying with or without human TAP1 or TAP 1+2 genes into same loci. Each group contains five mice. This treatment were boosted at day seven of tumor inoculation. After twenty-one days, all mice were sacrificed and the tumor sizes were measured. (A) tumor sizes for each group are shown. (B) statistical analysis of t-test shows that in VV-hTAP1 or VV-hTAP1+2 treated group mice tumor growth cannot be inhibited by body immune system comparing to VV-PJS-5 (vactor alone) group mice, one-side P value > 0.05.

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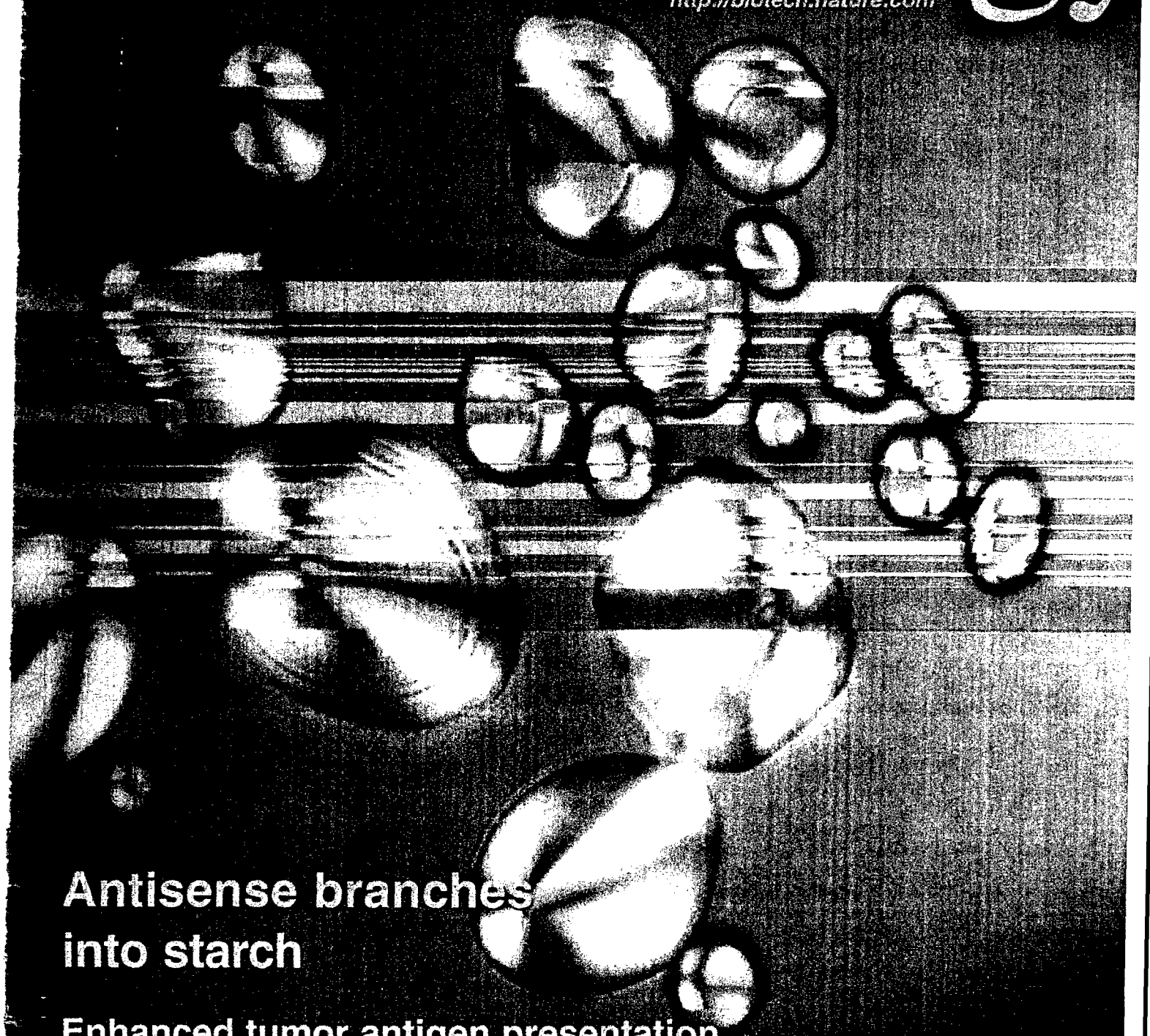

Antisense branches into starch

Enhanced tumor antigen presentation

Probing into proteasome activity

Expanding AAV packaging capacity

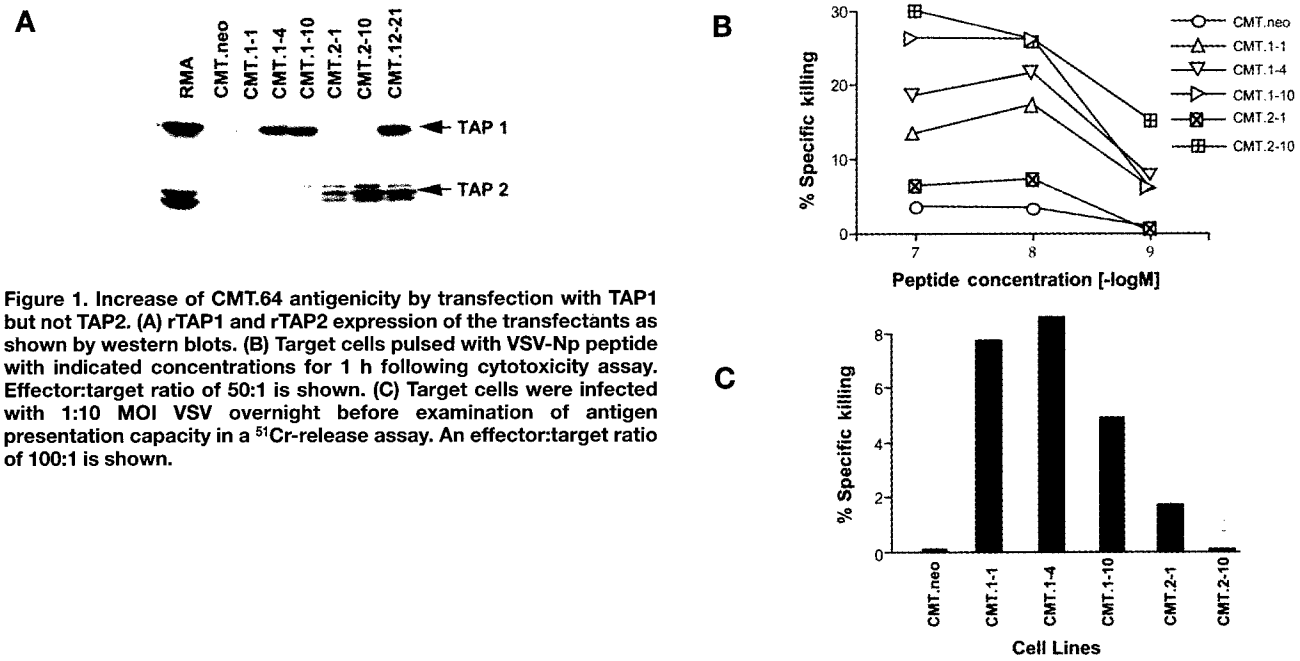

Figure 1. Increase of CMT.64 antigenicity by transfection with TAP1 but not TAP2. (A) rTAP1 and rTAP2 expression of the transfectants as shown by western blots. (B) Target cells pulsed with VSV-Np peptide with indicated concentrations for 1 h following cytotoxicity assay. Effector:target ratio of 50:1 is shown. (C) Target cells were infected with 1:10 MOI VSV overnight before examination of antigen presentation capacity in a ^{51}Cr-release assay. An effector:target ratio of 100:1 is shown.

the TAP-expressing tumor cells, destroy them, and thus prevent metastasis in vivo? To examine this possibility, we tested the TAP-deficient CMT.64, as well as TAP-transfected cell lines derived from CMT.64 in vivo, to determine whether TAP could improve the immune response against cancer cells and thus improve survival of animals bearing this tumor.

Results and discussion

Phenotype of TAP transfectants. The phenotype of the CMT.64 cell line has been described[18]. Transfection of rat TAPs (rTAP) into this cell line complements the expression of relevant components in the MHC class I-restricted antigen presentation pathway. Figure 1A shows transfectants expressing rTAP proteins. Two rTAP1-transfectant clones, CMT.1-4 and CMT.1-10, and one rTAP1,2-transfectant, CMT.12-21, express higher levels of rTAP1 protein than the CMT.1-1 clone (one of the rTAP1 clones). In comparison with RMA control cell line, these transfectants reveal a similar rTAP1 expression level (except CMT.1-1): CMT.1-1 clones express 10 times less, CMT.1-4 and CMT.1-10 express 2.5 times less, and CMT.12-21 expresses 2 times less. The rTAP2 expression levels in the transfectants were also examined in relation to RMA: CMT.2-1 and CMT.2-10 (two rTAP2-transfectant clones) express four times and two times less, respectively, and CMT.12-21 two times less. Thus, in comparison to RMA, all rTAP transfectants, except CMT.1-1, express similar levels of rTAP proteins.

TAP supplies peptides that bind to MHC class I molecules, resulting in their surface expression. Therefore, we examined MHC class I expression on the surface of transfectants by fluorescence-activated cell-sorting (FACS) analysis. Although TAPs were introduced into the CMT.64 cells, surface expression of MHC class I did not dramatically increase (Table 1), as judged by comparison to IFN-γ-treated CMT.64 cells, which restored high levels of MHC class I expression. This suggests that downregulation of MHC class I in CMT.64 cells likely occurs at the transcriptional level. However, constitutive expression of TAP restores some surface MHC class I expression (Table 1). It is noteworthy that the levels of MHC class I expression on the surface of TAP-transfectant clones quantitatively predict antigenic peptide binding. Cells pulsed with the immunodominant peptide derived from vesicular stomatitis virus nucleoprotein (VSV-Np) were killed equally well in a cytotoxic CTL assay, demonstrating that functional amounts of MHC class I are expressed on all transfectants, except CMT.neo and CMT.2-1 (Fig. 1B).

It is well known that the presentation of endogenously generated antigenic peptides to the cell surface for CTL recognition requires that peptides have the capacity to be transported by TAP and to bind to relevant MHC class I. An additional requirement is that there be sufficient quantities of peptides generated in the cytosol. Peptide-pulse experiments merely indicate whether surface MHC class I expression for CTL recognition is sufficient but do not confirm the overall antigen presentation capacity. Thus, we infected transfectants with VSV at a multiplicity of infection (MOI) overnight and of 10:1, then performed cytotoxicity assays. As shown in Figure 1C, the results demonstrated that three clones of the rTAP1 transfectant were able to present the immunodominant epitope, VSV-Np, whereas two rTAP2 clones and CMT.neo were unable to present this epitope efficiently. In a separate experiment, a clone transfected with rTAP1.2, CMT.12-21, also presented this epitope efficiently (data not shown). Our results suggest that only rTAP1 or rTAP1 and 2, but not rTAP2-transfected clones increase their antigenicity and acquire the ability to process and present foreign antigens. This difference cannot be attributed to levels of rTAP2 expression, since all TAP transfectants, except CMT.1-1 clone, express similar levels of TAP1 and/or TAP2 compared with RMA-TAPs. Taken together, antigen

Table 1. Comparison of MHC class I expression on surface of the CMT-TAP transfectants[a]

Cell line	D^b	K^b
CMT.64	18	0
CMT.neo	N.D.	0.1
CMT.1-1	N.D.	62
CMT.1-4	164	64
CMT.1-10	68	36
CMT.2-1	10	3
CMT.2-10	39	48
CMT.12-21	103	22
CMT.64 + IFN-γ	1,100	590

[a]Surface expression of D^b and K^b molecules was performed on the CMT.64 and its TAP transfectants by FACS analysis. The monoclonal antibodies, Y-3 (against K^b) and 28.14.8.S (against D^b) were used in this assay. The results are normalized by subtracting immunofluorescence intensity of negative control from each result. N.D., Not determined.

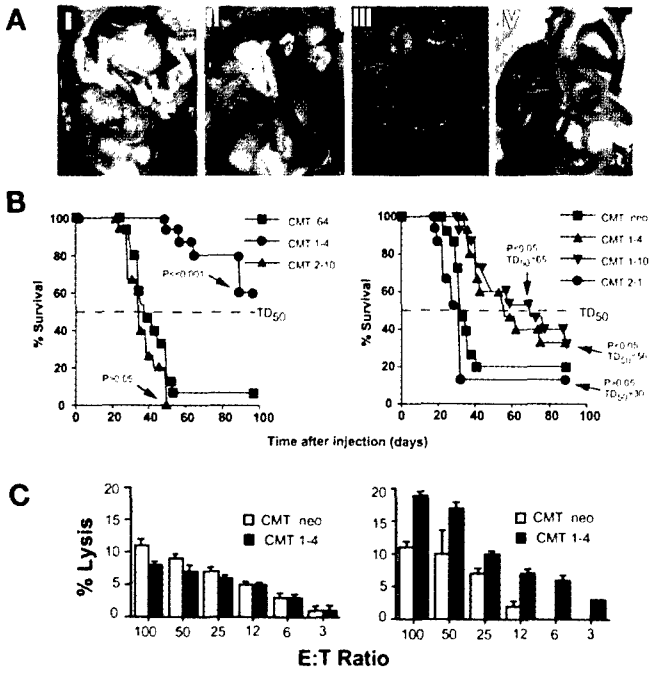

Figure 2. Control of tumor growth in vivo and improvement of mice survival by introducing rTAP heterodimer or rTAP1 but not rTAP2 into CMT.64 tumor cell line. CMT.64 or its transfectants were injected intraperitoneally into syngeneic mice. (A) After one month, one representative mouse from each group was sacrificed and the tumor growth pattern was examined. Panel I, CMT.neo; panel II, CMT.1-10; panel III, CMT.2-10; panel IV, CMT.12-21. The arrows indicate the tumors. (B) The time after injection of morbidity in mice was recorded for each group. Statistical analysis yields *p* value, comparing survival rates of CMT.64-bearing mice (left panel) or CMT.neo-bearing mice (right panel). (C) The specificity of splenocytes from a mouse injected with CMT.neo (left panel) or CMT.1-4 (right panel) was determined in a CTL assay against the targets CMT.neo and CMT.1-4.

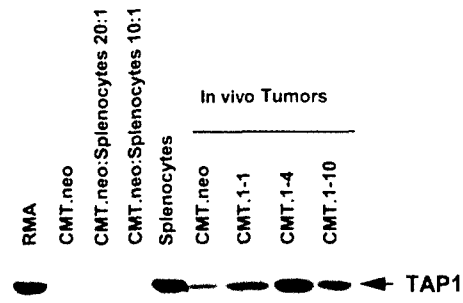

Figure 3. TAP1 expression within growing tumors in mice. TAP1 expression of in vivo tumors or cell lines was detected by western blot using D90 rabbit serum specific for rat and mice TAP1.

presentation appears to largely depend on TAP1 function or TAP1,2 heterodimer function in CMT.64 transfectants.

TAP1 improves immune recognition of tumors in vivo. Since in vitro experiments provide evidence that rTAP is able to improve specific CTL recognition, these results could be applied in immunosurveillance against tumors in vivo. To test this hypothesis, we first determined whether the host immune system could control the growth of CMT.64 transfectants. Mice were injected with CMT.neo or rTAP-transfected cells. On day 30 after injection, one representative mouse from each group was killed in order to examine the tumor growth pattern. The results are depicted in Figure 2A. Two rTAP2-transfected cell lines, CMT.2-1 and CMT.2-10 (see Fig. 2A, panel III for one example), had a tumor growth pattern identical to the control tumor, CMT.neo (Fig. 2A, panel I). Interestingly, in rTAP1 or rTAP1,2 transfectants, either tumors grew to form one large tumor (CMT.1-1 and CMT.1-10) (Fig. 2A, panel II, one example) or no tumor was present (CMT.1-4 and CMT.12-21) (Fig. 2A, panel IV, one example). Furthermore, on day 60, CMT.1-4 tumors demonstrated the same growth pattern as the other rTAP1-transfected clones (data not shown). These results suggest that tumors with rTAP1 or rTAP1 and 2 have limited tumor foci or are absent, whereas rTAP2-transfected tumors have the same level of metastasis as the wild-type tumors. This is true for other tumor-bearing mice (data not shown).

In a subsequent set of experiments, we addressed whether or not immune recognition of TAP1-transfected tumors could prolong the survival of tumor-bearing animals. Figure 2B depicts two independent experiments and summarizes the survival rates of mice injected intraperitoneally with CMT.64, or rTAP1- or rTAP2-transfected clones. At day 40–42 postinjection, 50% of CMT.64 and CMT.2-10 tumor-bearing group mice had died (Fig. 2B, left panel), and statistical analysis demonstrated no difference between these two groups ($p >> 0.05$). In contrast, after 100 days 60% of the CMT.1-4 group mice were still alive ($p << 0.001$) (Fig. 2B, left panel). To confirm that increased survival is not due to variation of rTAP1-transfected clones, in a repeated experiment with other clones, we also confirmed protection in another rTAP1-expressing clone ($p < 0.05$) but not rTAP2 ($p > 0.05$) (Fig. 2B, right panel). This demonstrates that this effect is not specific to a single TAP1-expressing clone. Autopsy examination of all mice represented in Figure 2B revealed the patterns noted Figure 2A. As an experimental control, no difference was observed between CMT.64 and CMT.neo cell lines (data not shown). Our results suggest that improvement of the survival rates of rTAP1 tumor-bearing mice may be due to enhancement of the tumor's immunogenicity, which in turn would trigger the antitumor immune response of the hosts.

The nature of tumor recognition. T cells are critical factors in the defense against the development of most tumors. The presence of lymphocytic infiltrates within many malignant tumors has been suggested to indicate an in vivo antitumor immune response[20]. Since

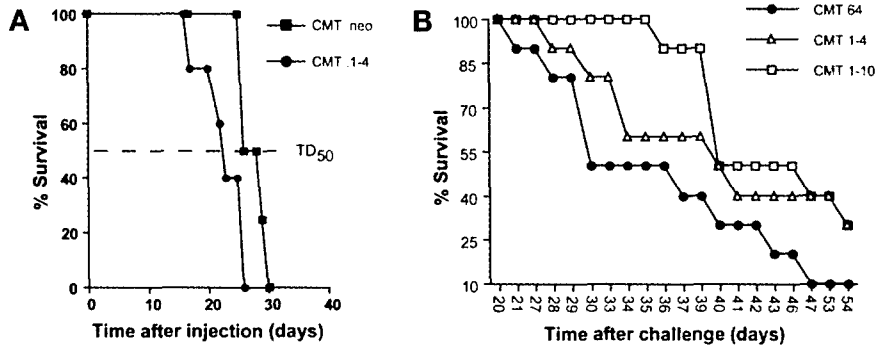

Figure 4. Examination of the survival of mice bearing CMT tumors. (A) rTAP1 does not improve recognition of CMT.64 in nude mice. A total of 5×10^5 cells of either CMT.1-4 (five mice) or CMT.neo (four mice) were injected intraperitoneally into nude mice (H-2^b). The time of morbidity was recorded for each group. (B) Immunization with rTAP-transfected tumors improves the survival of CMT.64-bearing mice. A total of 1×10^7 cells of CMT.neo, CMT.1-4, or CMT.1-10 were treated with mitomycin C (30 mg ml^{-1}) for 2 h and γ-irradiated before intraperitoneal injection into 10 C57BL/6 mice. One month later the mice were challenged intraperitoneally with 5×10^5 CMT.64 cells in PBS. The time of morbidity was recorded.

Table 2. Percentage of tumor-infiltrating lymphocytes within growing tumors in mice[a]

Tumor	One month		Two months		Ratio[b]	
	CD4 %	CD8 %	CD4 %	CD8 %	CD4	CD8
CMT.neo	0.54	0.41				
CMT.1-1	1.02	0.87			1.9	2.1
CMT.1-4			1.07	1.20	2.0	2.9
CMT.1-10	4.19	2.94			7.8	7.2
CMT.2-1	0.24	0.23			0.4	0.6
CMT.2-10	0.12	0.66			0.2	1.6

[a]CD4 and CD8 T cells in tumors were detected by FACS analysis using monoclonal antibodies, RM4-5 (against CD4 or CD8 positive cells) and 53-6.7 (against CD8). % = 100% × number total cells (including tumor cells).
[b]Ratio = CD4 % or CD8 % of TAP transfectants/CD4 % or CD8 % of CMT.neo.

in vivo protection from rTAP1 tumors is controlled by the host immune system, we compared the percentage of tumor-infiltrating lymphocytes (TILs) between different tumors. We examined TILs in animals one month or two months after injection of CMT.64 transfectants using flow cytometry. The results are shown in Table 2. The ratios of $CD4^+$ and $CD8^+$ T cells were enhanced by two to eight times in rTAP1 tumors compared with control, CMT.neo, and rTAP2 tumors.

The observed increases of TIL in TAP1 tumors suggest the presence of specific cytolytic T cells. This possibility is based on three lines of evidence: (1) TAP1 increases tumor surface MHC class I; (2) TAP1 improves antigen presentation; (3) TAP1 results in tumors being controlled in vivo and improves animal survival. If CMT.64 cells contain a TAA, then specific CTLs should be generated by antigen presentation in rTAP1 transfectants in vivo. We performed CTL analysis using splenocytes from mice immunized with CMT.neo or CMT.1-4. CMT.neo-stimulated and CMT.1-4-stimulated splenocytes were compared against CMT.neo and CMT.1-4 targets in a standard ^{51}Cr-release assay. Splenocytes from CMT.1-4-immunized mice were much better than splenocytes from CMT.neo mice at killing target cells (Fig. 2C). Killing of CMT.neo and CMT.1-4 targets by CMT.neo splenocytes was equivalent (Fig. 2C, left panel). In contrast, killing by CMT.1-4-stimulated splenocytes of CMT.1-4 targets was enhanced at both high and low effector:target (E:T) ratios (Fig. 2C, right panel). These results suggest that CMT.1-4 cells contain an antigenic antigen(s), TAA, and that this antigen can be presented by TAP1-expressing tumors, triggering host T-cell recognition.

The importance of T cells in antitumor immunity has been further confirmed by using athymic mice, which are devoid of T lymphocytes[21]. Unlike wild-type animals, the survival rates of athymic mice were not significantly different between CMT.neo- and CMT.1-4-bearing mice groups (Fig. 4A).

Immunization with rTAP1-transfected cells affords protection against wild-type tumor cells. Having confirmed that rTAP1-transfected cells possess antigenicity and immunogenicity, we were interested in testing whether or not immunization with TAP1-positive cells affords protection against TAP-deficient CMT.64 cells. Three groups of mice were immunized intraperitoneally with mitomycin- and irradiation-treated cells: CMT.neo, CMT.1-4, and CMT.1-10, respectively. One month later the mice were challenged intraperitoneally with wild-type CMT.64 cells, and then monitored for survival. The results are shown in Figure 4B. Mice immunized with both immunogenic rTAP1-transfected clones show a successful challenge with TAP-negative CMT.64, compared with CMT.neo immunization. A statistical analysis of regression on logarithmic percentage survival showed this effect to be significant ($p < 0.001$ for CMT.1-10 immunization; $p < 0.05$ for CMT.1-4 immunization.

Successful challenge of wild-type tumors following immunization with rTAP1 but not CMT.neo tumor cells suggests that the immunization with immunogenic tumors can augment the antitumor response. Obviously, specific T cells participate in this immune response. Although it is not clear how T cells, especially CTLs, directly recognize wild-type CMT.64 cells, in vivo they, along with other immune elements, may compose the integral components of the specific antitumor response.

Analysis of rTAP1 expression in in vivo growing tumors. At this point we became curious whether the rare large tumors seen in mice receiving the TAP1 transfectants had lost TAP expression, perhaps hastened by selective pressure by the host immune system. A western blot analysis was performed on the rTAP1 tumors that had grown in mice for one month or two months. Tumors, in vivo, contain normal mouse cells (such as CD4 and CD8 T cells) that express mouse TAPs. Our TAP1-specific antibody recognizes both mouse and rat TAP1, and it was initially very difficult to judge the expression of rTAP1 in these solid tumors. We, therefore, included controls that consisted of mixing CMT.neo cells with a 20:1 or 10:1 ratio of the wild-type splenocytes from the mice. The results are shown in Figure 3. The CMT.neo tumor contained a stronger TAP1 signal than the CMT.neo–splenocytes mixture at a 10:1 ratio, suggesting that other types of wild-type mouse cells, excluding T cells (CMT.neo tumor contains ~1% T cells; see Table 2), had infiltrated the tumor. As expected, in comparison with rTAP1 tumor signals, the CMT.neo tumor's signal was much less (see Fig. 3). Although we cannot judge whether the amount of mouse wild-type infiltrating cells induced

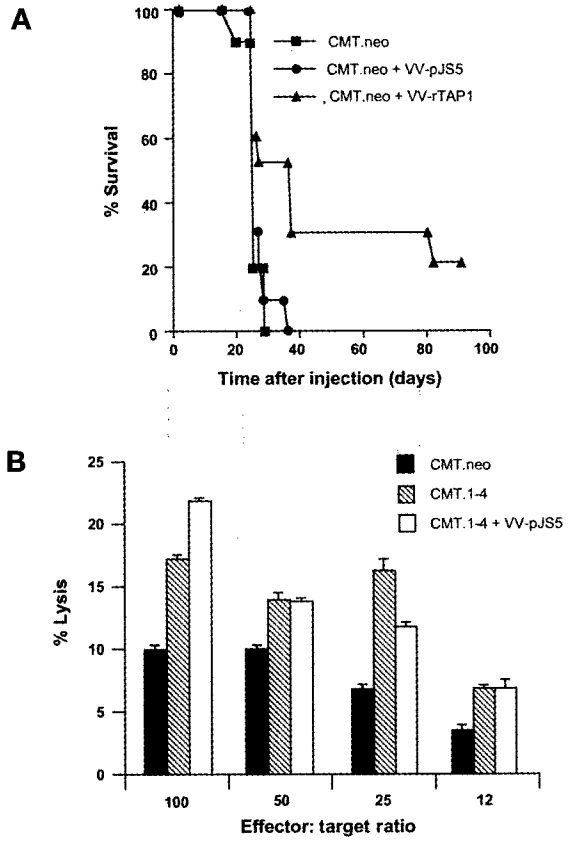

Figure 5. Antitumor immune therapy by VV-rTAP1. (A) Each group of mice was injected intraperitoneally with CMT.neo cells. Two out of three groups subsequently received treatments of either VV-pJS5 or VV-rYAP1 with 10^6 p.f.u. in PBS containing 2% mouse serum at 24 h and two weeks after cell injection. Control group received only PBS containing 2% mouse serum. Statistical analysis shows that VV-rTAP1 treatment of tumor-bearing mice has a significant p value ($p < 0.05$), comparing with both VV-pJS5 and mimic treatment. (B) The specificity of splenocytes from two mice injected with CMT.neo and VV-pJS5 infected CMT.1-4 (10:1 MOI infection for 3.5 h).

the differences in levels of TAP expression between CMT.neo tumor and rTAP1 tumor clones (CMT.1-1 and CMT.1-10), the intensity as judged by gel scanning of TAP1 in CMT.1-4 tumor is identical to that in RMA cells. Since the CMT.1-4 cells express half the amount of rTAP1 protein compared to the RMA cells (see Fig. 1A), and this tumor, in vivo, contains fewer T cells than CMT.1-10 tumor (see Table 2) (suggesting less contamination by wild-type infiltrating cells), we conclude that the TAP-transfected tumors maintain the expression of rTAP1 during two months' growth in vivo.

Our data suggested that the large tumors are not TAP revertants but also implied a more interesting possibility. The TAP1 levels between the different TAP-expressing clones generally appeared to correspond with the malignancy of the tumor. The more TAP1 expressed, the fewer the number of tumors observed. These data lead us to speculate that the reason we see some large tumors in the TAP1 expressers is that the TAP1 expression levels are too low to provide complete protection. Alternatively, in a naive mouse the initial tumor burden has formed a solid focus, leading to a late-stage metastatic carcinoma that cannot be controlled by a specific antitumor immune response. The latter possibility is supported by our results, which showed that all surviving mice that were initially challenged with live rTAP1 or rTAP1,2 tumor cells (nonirradiated) remained healthy after subsequent challenge with rTAP1 tumor cells (data not shown).

Contribution of TAP1 to cancer therapy. We have shown that TAP1 improves CMT.64 immunogenicity and host survival rates. This has led us to explore whether TAP1 can form the basis of a tumor immunotherapy. An expression vector of recombinant vaccinia virus carrying rTAP1 gene (VV-rTAP1) was generated for these experiments. A faithful model for viral therapy for tumor-burdened individuals entails infection in vivo after the tumor load had been established. To examine this scenario, 5×10^5 CMT.neo cells were injected into three mouse groups. After 24 h, mice received either 10^6 p.f.u. VV-rTAP1, VV-pJS5 (control vector), or phosphate-buffered saline (PBS) containing 2% C57BL/6 mouse serum. This procedure was performed again at two weeks. As expected, the vector alone, VV-pJS5, did not increase mouse survival, as judged by comparison to the PBS group ($p >> 0.05$) (see Fig. 5A). However, the mice receiving VV-rTAP1 treatment had a significantly higher survival rate, as judged by comparison to the PBS group ($p << 0.05$) and the VV-pJS5 group ($p << 0.05$) (see Fig. 5A).

We sought to confirm that the improved survival rate of tumor-bearing mice was due to the host immune system recognizing antigens, including tumor antigens, after VV-rTAP1 infection. We performed CTL analysis by using the splenocytes from mice injected with CMT.neo plus VV-rTAP1. The targets were CMT.neo, CMT.1-4, and CMT.1-4 infected with 10:1 MOI VV-pJS5. If the splenocytes contained TAA-specific CTLs, then they would kill CMT.1-4 targets and, therefore, confirm the presentation in vivo of tumor antigens in VV-rTAP1-infected CMT.neo. The results are shown in Figure 5B. In comparison with control CMT.neo, CMT.1-4 targets were killed more efficiently, suggesting that tumor antigens are presented in VV-rTAP1-infected CMT.neo tumors in vivo. In addition, these results also show that CMT.1-4 infected with VV-pJS5 achieved levels of killing equal to CMT.1-4, suggesting the splenocytes also contained VV-specific T cells. Thus, viral-infected tumor cells provide additional antigen(s) to the host immune system for recognition. Taken together, we conclude that the improved mouse survival is likely due to the presentation of both tumor and VV antigens after the viral therapy.

Introducing TAP into the tumor-burdened individuals by using a vector provides a possible method for antitumor immune therapy. It has a potential significance for controlling the occurrence of neoplastic metastasis and for treating metastasized neoplasms. This would be particularly true for the tumors with a deficiency in components of the antigen-processing machinery. Utilizing VV allows TAP to be produced in any cell of the body, and using TAP in this manner provides a possible treatment for improving survival. The use of VV as gene-delivering system is not the only choice; indeed, many methods of expressing specific proteins in different cancer cells have been developed. Whichever gene-delivering system is used, targeting TAP into cancer cells exclusively is not likely to be necessary, unless evidence is generated suggesting that a risk exists of developing autoimmunity. However, we observed no increased autoimmunity in this system (manuscript in preparation). Tumors that down-regulate their antigen-presenting capabilities successfully evade the immune system. To allow recognition of these tumors, one would have to match the polymorphism of the MHC with the binding and immunogenicity of allele-specific peptide expressed by different tumors. By acting independently of the tumor antigen and MHC polymorphism, TAP vaccines bypass these requirements to increase recognition.

Further investigation is necessary to determine the effects of this treatment on other types of tumors and whether the results seen in this experiment will ultimately translate into a therapy for human disease.

Experimental protocol

Animals. The mouse strain C57BL/6 (H-2^b) was obtained from Jackson Laboratories but housed and bred by Willem Schoorl at the Biotechnology Breeding Facility (University of British Columbia). The H-2^b nude mice (B/6 Nu-M (C57BL/6 NTAC-NufDF)) were obtained from Taconic (Hanover, NY) and kept in specific pathogen-free incubators. The mice were maintained according to the guidelines of the Canadian Council on Animal Care. The mice used in the experiments were between 6 and 12 weeks of age and were killed by CO_2 asphyxiation.

Recombinant vaccinia virus (VV) construction. Recombinant VV was constructed by homologous recombination of the wild-type VV WR strain by infecting CV-1 cells transfected with the plasmids pJS5, pJS5-rTAP1, pJS5-rTAP2, or pJS5-rTAP1,2 according to described protocols[22].

Purification of VV stocks. Crude cell stocks were used for the infection of cells in culture, but purified stocks of VV were used when injecting mice. To purify the VV, 3 L batches of VV-infected Hela S3 cultures were used. The VV was released from the cells by homogenization with a Dounce homogenizer before centrifugation at 750 *g* for 5 min at 4°C. The supernatant was trypsinized with 0.1 volume of 2.5 mg ml^{-1} trypsin for 30 min at 37°C, then layered onto an equal volume of 36% sucrose in 10 mM Tris-HCl pH 9.0. It was centrifuged for 80 min at 4°C at 25,000 *g* and the pellet was then resuspended in 1 mM Tris-HCl pH 9.0. The pellet was trypsinized again before being layered onto a 24–40% continuous sucrose gradient and centrifuged for 45 min, at 4°C at 18,750 *g*. The milky band was collected and saved, whereas the pellet was trypsinized and repurified on another sucrose gradient. All of the bands collected were pelleted by diluting with two volumes of 1 mM Tris-HCl pH 9.0 and centrifuging for 60 min at 4°C at 25,000 *g*. The viral pellet was resuspended in 1 mM Tris-HCl pH 9.0, and 0.5 ml aliquots were stored at -80°C or -135°C.

Tissue culture. The small-cell lung carcinoma cell line, CMT.64, used in the cancer experiments originated spontaneously from the C57BL/6 mouse strain[15]. All of the stable CMT.64 transfectants containing rTAP-1 (CMT.1-1, CMT.1-4, CMT.1-10), rTAP-2 (CMT.2-1, CMT.2-10), rTAP1,2 (CMT.12-21), and the vector-only control (CMT.neo) were created by transfecting CMT.64 cells with the rTAP cDNA in mammalian expression vector pH(Apr-1neo)[18,19]. All cell lines including the RMA cell line were grown in either Dulbecco's modified Eagle's medium (DMEM) or RPMI containing 10% fetal bovine serum (FBS).

Generation of effector cell populations. Virus-specific CTL populations were generated by infecting mice intraperitoneally with 10^7 tissue culture infection dose (TCID) units of VSV or at the suggested plaque-forming units (p.f.u.) for VV-TAP or VV-pJS5 vector. Cytolytic T lymphocytes were collected on day 5 postimmunization from the cervical lymph nodes (LN) or spleen and cultured in RPMI-1640 medium containing 10% FBS, 20 mM HEPES, 2 mM L-glutamine, 0.1 mM essential amino acids, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol (β-ME), and penicillin/streptomycin (henceforth referred to as RPMI complete medium). The LN cell

suspensions were cultured at 4×10^6 cells/ml for three to five days in the absence of stimulation before being used in a CTL assay, whereas the splenocyte suspension was cultured for seven days with peptide stimulation. Bulk populations of VSV-specific CTLs were maintained by weekly restimulation with 1 μM VSV-Np (amino acids 52–59) plus pulsed irradiated (2,200 rads) stimulator splenocytes. Irradiated stimulator cells and CTLs were incubated together at a ratio of 4:1 in RPMI complete medium containing 20 units ml^{-1} human interleukin 2 (hIL-2). Seven days later, this bulk population was used in a CTL assay.

For antitumor CTL generation, the specificity of splenocytes was generated by injecting mice intraperitoneally with 1×10^7 CMT.neo or CMT.1-4 cells (Fig. 2C). Upon removal the splenocytes were cultured with stimulators at a 3:1 ratio. The stimulators were prepared by incubating CMT1-4 or CMT.neo cells with 30 mg ml^{-1} mitomycin C under hypoxic conditions. After incubation for 2 h the cells were γ-irradiated (10,000 rads) and washed three times before addition to the splenocyte culture. CMT.neo splenocytes received CMT.neo stimulators, whereas CMT1-4 splenocytes received CMT1-4 stimulators. Six days after in vitro stimulation the splenocytes were tested in a standard 4 h ^{51}Cr-release assay.

For antitumor and VV CTL generation, the specificity of splenocytes was generated by injecting mice intraperitoneally with 1×10^7 CMT.neo cells and 1×10^6 p.f.u. VV-rTAP1 (Fig. 5B). The splenocytes were a secondary mass culture that were incubated with stimulator cells, plus γ-irradiated (5,000 rads) naive syngeneic splenocytes, at a 5:1:15 ratio. The stimulator cells were prepared by infecting CMT.neo cells with VV-rTAP1 for 3 h before adding 30 mg ml^{-1} mitomycin C under hypoxic conditions. After incubation for 2 h the cells were γ-irradiated (10,000 rads) and washed three times before addition to the splenocyte culture. After incubation for six days the splenocytes were tested in a standard 4 h ^{51}Cr-release assay.

Cytotoxicity assays. Target cells for the CTL assays were loaded with ^{51}Cr by incubating 10^6 cells with 100 μCi of ^{51}Cr (as sodium chromate; Amersham, Arlington Heights, IL) in 250 μl of CTL medium (RPMI-1640 containing 10% (vol/vol) HI FBS, 20 mM HEPES) for 1 h. Following three washes with RPMI, 2% (vol/vol) FBS, the target cells were incubated with the effector cells at the indicated ratios for 4 h. 100 μl of supernatant from each well were collected and the ^{51}Cr release was measured by a γ-counter (LKB Instruments, Gaithersburg, MD). The specific ^{51}Cr release was calculated as follows: ((experimental - media control) / (total - media control)) × 100%. The total release was obtained by lysis of the cells with a 5% Triton X-100 (BDH) solution.

FACS assays. Surface expression of the H-2K^b allele was detected by indirect immunofluorescence using the conformational-dependent mouse monoclonal antibodies AF6-88-5.3 (ATCC, Manassas, VA) and 142.23 (a gift from Dr. Sun Kvist), both specific for the complex of K^b–β_2M. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Dakopatts, DK) was used as the secondary antibody. The mean logarithmic fluorescence intensity was measured by a FACScan analyzer (Becton Dickinson, Mountain View, CA). For detection of $CD4^+$ T cells and $CD8^+$ T cells we used a protocol similar to that used for detection of surface MHC class I molecules, with minor modifications. Briefly, tumors were washed extensively and homogenized into single cells. FITC-conjugated rabbit anti-mouse antibodies (BD PharMingen, San Diego, CA) RM4-5 (against CD4) and 53-6.7 (against CD8) were used.

Western blots. The proteins of lysates from 5×10^5 cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% resolving gel, and then were transferred to a nitrocellulose membrane. The blots were probed with either the rabbit anti-rat TAP1 (D90) or TAP2 (114/2) polyclonal antibody at a dilution of 1:1,000, and then incubated with horseradish peroxidase-labeled anti-rabbit antibody at a 1:100,000 dilution. The immunocomplexes were visualized by enhanced chemiluminescence (ECL) according to the instructions of the manufacturer (Amersham) and were quantitatively assessed by a densitometry scan.

Inoculation of mice with tumor cell lines. A total of 5×10^5 (unless otherwise indicated in figure legend) cells of CMT.64 or its transfectants in PBS were injected intraperitoneally into C57BL/6 syngeneic mice. For examination of tumor growth pattern, one representative mouse from each group (four mice for each group) was killed after one month for photographing of in vivo tumors (Fig. 2A). For mice survival experiments, each group contained 15 (Fig. 2B) or 10 (Fig. 4B and 5A) mice.

Statistics. The statistics for the cancer studies were performed using the Kaplan–Meier log rank survival test or regression log percentage survival test before carrying out a paired *t*-test. The computer software program JMP IN version 3.2.1 was used to do the computations[23]. The data were considered statistically different if $p < 0.05$.

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NEWS AND VIEWS

Problem solving for tumor immunotherapy

Cécile Gouttefangeas and Hans-Georg Rammensee

Tumor cells express defined antigens that can be recognized by tumor-destroying ($CD8^+$) cytotoxic T lymphocytes (CTLs). As most cancer patients obviously do not mount efficient T-cell responses against their tumors, the task is clear: immunotherapies must induce cancer-destroying T cells in patients. Although this goal appears straightforward, effective immunotherapy has remained elusive because of three major problems: first, for many tumors, no or not enough suitable antigens are known; second, no consensus exists for the best antigen formulation or the route of immunization; and third, tumors under immune attack tend to be selected for antigen loss variants. These three problems lie at the heart of two studies[1,2] published in this issue and one published recently in *Nature Medicine*[3].

Currently, problem number one—that of identifying specific tumor antigens—is closest to solution. An increasing number of antigens are being found by screening for gene products differentially expressed in tumors as opposed to normal tissues and by testing for antigenicity. In addition, vaccination trials are underway with preparations containing multiple unidentified tumor antigens, such as heat shock proteins isolated from autologous tumors or allogeneic dendritic antigen-presenting cells (APCs) fused with autologous tumor cells, the latter with surprising success[3].

Problem number two—optimizing antigen presentation and delivery—is more complex, as illustrated by the high number of antigen formulation and immunization modes and routes currently employed in clinical trials. Antigen formulations can take the form of peptides, proteins, DNA, RNA, viral vectors, modified cells either alone or together with adjuvants, cytokines, or in vitro-derived dendritic cells. The approach used by Cho et al.[1] is a new combination of a protein antigen coupled to adjuvant-like immunostimulatory DNA.

Problem number three—loss of antigen—can be potentially avoided if immunization is carried out at an early stage in the disease and with multiple antigens. If this fails, it may be possible to restore antigen expression, as suggested by Alimonti et al.[2].

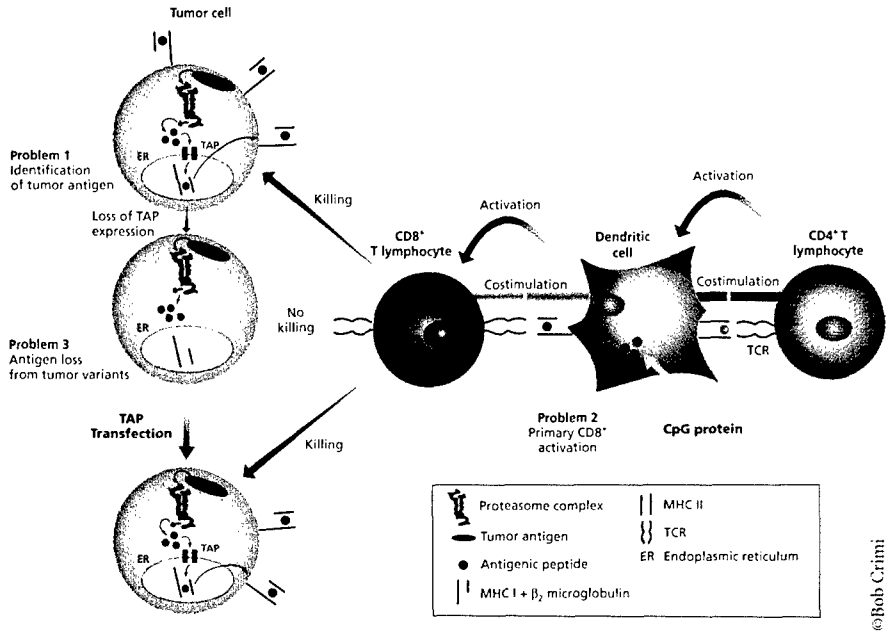

Figure 1. Three main problems in inducing $CD8^+$T lymphocytes to kill tumor cells in vivo: Efficient activation of the CTL needs interaction with an activated dendritic cell (problem number 2). Identification of new tumor antigens and dealing with antigen loss variants are also critical points for improving immunotherapy protocols in cancer patients (problems number 1 and 3). Red arrows show two examples of strategies that may be used to boost CTL respopnses against tumors, as described by Cho et al. and Alimonti et al. in this issue.

To understand the different strategies employed, it is necessary to outline briefly the way in which a CTL attacks a tumor cell. $CD8^+$ CTLs specifically recognize short peptide fragments presented by major histocompatibility complex (MHC) class I molecules at the cell surface of target cells. The process of protein degradation into peptides in the cytosol, their assembly with newly synthesized MHC proteins in the endoplasmic reticulum, and export to the cell surface is called antigen processing[4]. The peptides recognized by CTLs are derived either from viral proteins if the cells are infected, or from aberrantly expressed proteins in tumor cells. Thus, tumor-associated proteins that are solely expressed intracellularly are still visible for CTLs (but not antibodies) by virtue of MHC class I-associated peptide presentation.

However, cancer cells are poorly immunogenic themselves. In vivo CTL induction requires contact with a professional APC, such as the dendritic cell, which first takes up the tumor antigen and is activated itself by interactions with $CD4^+$ helper T cells (see Fig. 1)[5]. This leads to the increased expression of proteins from the B7 family and adhesion molecules, all of which are essential for the efficient co-stimulation and activation of T lymphocytes. If successfully activated, the $CD8^+$ CTLs kill the tumor cells as long as the antigen continues to be expressed.

Unfortunately, escape mutants of tumor cells also commonly arise. Apart from deletion of the antigen itself, one or several proteins of the antigen presentation pathway can become defective; this includes the peptide transporter TAP, subunits of the proteasome complex, or even class I components themselves (see Fig. 1)[6].

In this issue, Cho et al. report a new vaccine using DNA that, rather than encoding an antigen, acts as an adjuvant of a protein antigen. DNA-based vaccination of mice using a DNA sequence encoding the antigen and additional "immunostimulatory sequences"

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containing unmethylated cytosine/guanosine-rich motifs (CpG) have already been shown to induce antigen-specific CTLs[7]. Cho et al. have now coupled CpG sequences covalently to a well-known model protein, ovalbumin, and shown that this construct induces CTL activity against ovalbumin-expressing target cells upon injection into mice. Surprisingly, CTL induction by the CpG–protein construct is independent of $CD4^+$ help, as demonstrated by CTL activity in CD4 knockout mice. Vaccination with the construct protects against pre-established ovalbumin-expressing tumors and is mediated by $CD8^+$ effector cells.

There are two remarkable features in this new combination of DNA-adjuvant and protein antigen: First, the CpG sequences seem to direct the presentation of exogenous antigen to the class I pathway, although the mechanism implicated is unknown (see Fig. 1). Second, the construct bypasses $CD4^+$-mediated help, most likely by providing both a "danger" signal and differentiation signals directly to dendritic cells, as demonstrated recently for CpG–peptide mixtures in mice[8]. In addition, CTL induction with the CpG–protein construct is much more efficient than with other ovalbumin preparations.

Although this all sounds very straightforward, we do not know yet whether such constructs work in humans. Some immunostimulatory effects of CpG motifs have been described in human peripheral blood in vitro, most notably in dendritic cells[9], but immunization trials have not been reported. Thus, the efficacy of CpG–protein constructs for immunotherapy in patients remains to be tested.

Once efficient tumor-destroying T cells are induced, tumor cells may escape by antigen loss, as mentioned above. One way of achieving this is to downregulate TAP activity[6].

In a second paper, Alimonti et al. explore the possibility of restoring antigenicity by transferring one of the TAP genes into a TAP-deficient tumor cell via transfection or infection with a TAP1-containing vaccinia virus construct. The results suggest a beneficial effect, although it is unclear how only one of the two TAP chains can restore TAP activity (maybe TAP1 induces marginal intrinsic TAP2 expression?).

Overall, their data suggest that reintroduction of one deficient component of the antigen-processing machinery (i.e., TAP1) may be enough to restore the peptide-presenting capability and the in vivo antigenicity of certain cancer cells. Since virus-mediated gene delivery is not tumor-specific, potential induction of autoimmunity by increasing the activity of the antigen-processing machinery in normal cells must be considered.

To date, vaccinations with peptides, peptides loaded onto dendritic cells, recombinant or tumor-derived proteins, or modified cells have proved efficacious only in certain patients. Thus, a powerful and reliable method of immunizing patients is still sought. A decade's worth of clinical trials for antigen-specific cancer immunotherapy suggests that optimization of antitumoral effector cell stimulation, possibly combined with improvement of tumor antigenicity by gene transfer or cytokines, may provide the best option. As the papers in this issue illustrate, there may be many approaches to solving the same problem.

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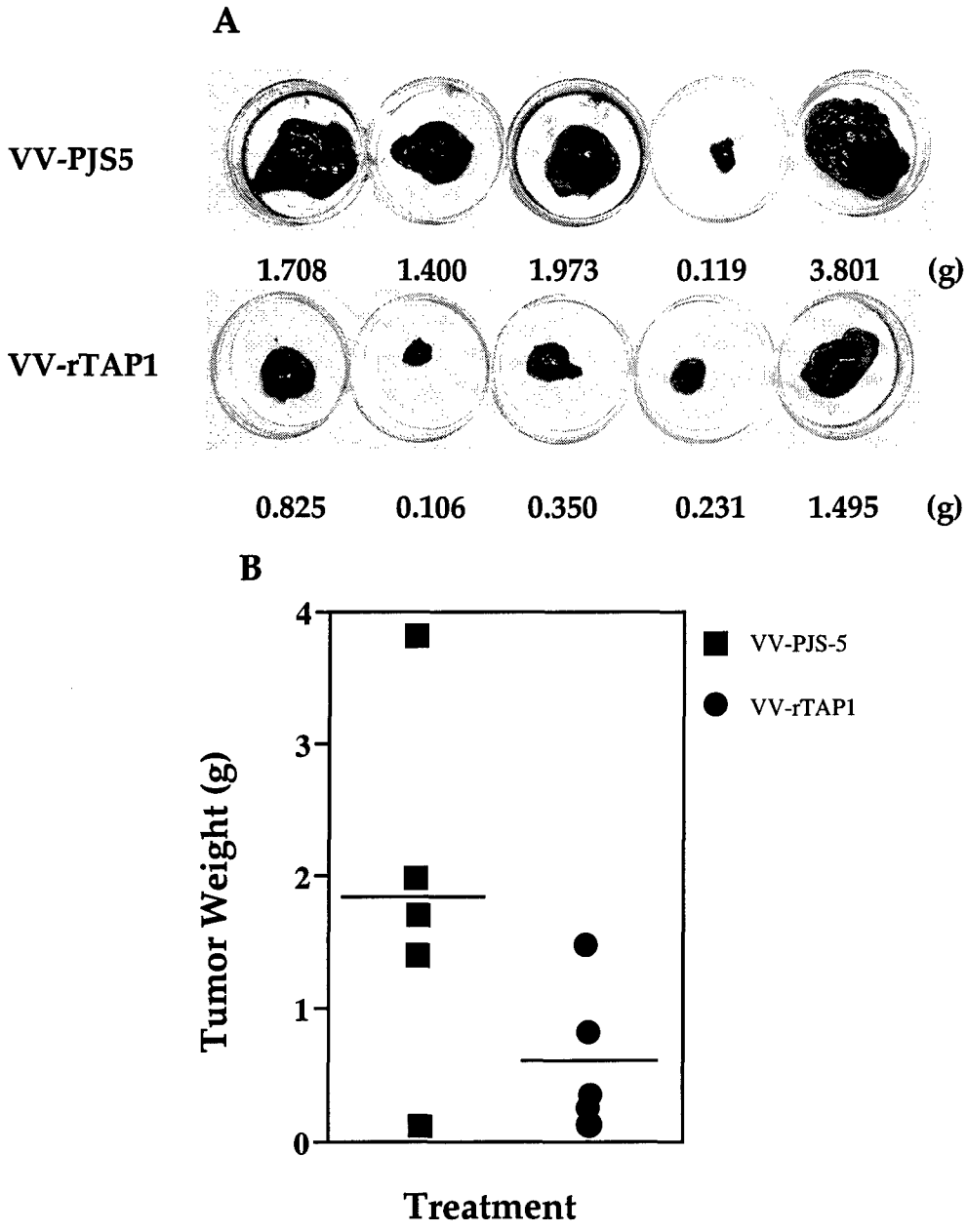

Appendix II. Rat TAP1-vaccinia virus infection significantly reduce B16F10 tumor growth in mice. $1.5X10^5$ B16F10 cells/mouse were subcutaneously (s.c.) injected into C57/BL6 mice. One day after, the mice received s.c.$1X10^6$ (PFU) of either vaccinia vactor alone (VV-PHS5)(5 mice/group) or vaccinia-carrying rat TAP1 (VV-rTAP1) (5 mice/group), and this procedure was repeated 7 days later. All mice were scarified at day 17th and, the tumor sizes are shown in (A) and mean value difference of tumor weights between two groups are shown in (B). The statistics of t-test shows that the one-sided P value is <0.05, indicating that the mice receiving VV-rTAP1 treatment the mean value of tumor weights is significantly less than that of control group.

Over-expression of TAP augments immune responses in normal mice

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Abstract

In order to elicit immune responses to viruses, virally-derived antigens must be presented to cytolytic T lymphocytes by MHC class I molecules on the cell surface of virally infected cells. The transporter associated with peptide processing or TAP is an integral protein complex of the antigen processing and presentation pathway. The rate of transport of MHC class I/peptide to the cell's surface is dependent upon the efficiency of peptide transfer into the ER by TAP and loading of 'empty' MHC class I molecules [1,2]. Thus, we reasoned that TAP expression could be a limiting factor in augmenting immune responses against viruses. In order to test this hypothesis, TAP was over-expressed in normal mice to determine if it could increase antigen presentation and therefore enhance antigen specific cytotoxic responses against whole virus. Evidence is provided that *in vivo* TAP is a limiting factor in viral responses and its over-expression can augment T cell immune responses. This is the first demonstration that over-expression of an intracellular component of the antigen processing machinery can augment immune responses in healthy animals. We conclude that TAP over-expression can act as an adjuvant and may provide a new general approach for increasing responses against virus in immunocompetent and immunocompromised hosts.

Results and Discussion

The TAP complex is responsible for maintaining the supply of peptides to newly synthesized MHC class I molecules. It has been suggested that the supply of peptides by TAP may be a limiting factor for the expression of stable MHC class I/peptide complex on the cell surface [1,2]. In the absence of TAP 'empty' MHC class I are retained in the ER. If increasing TAP expression in antigen-presenting cells (APCs) could increase the number of the immunogenic peptide-loaded MHC class I molecules on the cell surface, then perhaps T cell-mediated immune response could be augmented.

A subunit vaccine containing a TAP-expressing cassette was used in order to investigate this hypothesis. This system was created using recombinant vaccinia virus carrying human TAP1 and TAP2 genes [3] designated as VV-hTAP1,2. A cytotoxicity assay was performed to test TAP activity of the TAP-delivery system, as judged by the restoration of antigen presentation in a TAP deficient cell line, T2/K^b. This cell line, which lacks both TAP molecules [4,5] was transfected with the H-2K^b gene. As we expected, T2/K^b infected with VV-hTAP1,2 restored H-2K^b-restricted, VSV-Np52-59 epitope-specific, antigen presentation (Figure 1), indicating that the TAP transporter is fundamentally expressed from this expression system.

An experimental protocol was developed to directly assess whether TAP expression would augment immune responses against the wild-type virus. A viral dose that is required to generate a specific T cell population with minimal cytolytic activity was first established by titration of the VSV virus inoculum. We used this minimal dose of virus to generate a specific anti-VSV CTL response and

then tested whether this response can be augmented by co-inoculation of mice with VV-hTAP1,2. The immune response was monitored by performing an *in vitro* cytotoxicity assay using viral peptide-pulsed RMA cells as targets and specific CTL derived from the inoculated mice. In figure 2, we demonstrate that different doses of the inoculated VSV virus elicited varying levels of primary immune responses in naîve mice. Under our working conditions, a viral dose of 3.6×10^4 ($TCID_{50}$) units resulted in approximately a 10% maximal CTL response. Higher doses resulted in higher CTL responses and maximal activity was achieved at a viral inoculum of 3.6×10^6 $TCID_{50}$ units (Figure 2). The results may reflect the fact that CTL priming requires a threshold amount of relevant viral peptides expressed on the surface of antigen-presenting cells (APCs) [6,7]. These peptides must have undergone competition with self-derived peptides for transport into ER-lumen and MHC class I binding, before reaching the cell surface [8,9]. Hence, it is reasonable to believe that some viral peptides in low concentrations are excluded from the ER-lumen by this competition. At lower doses of virus inoculation, a parallel increase of TAP expression in antigen-presenting cells may allow more of the under-represented antigenic viral peptides to gain access to ER-lumen for MHC class I binding and surface expression and augment priming of specific CTLs. In figure 3A we demonstrated that co-injection of 3×10^4 (pfu) dose of vaccinia virus carrying TAP1 and TAP2 cDNAs (VV-hTAP1,2) with 3.6×10^4 ($TCID_{50}$) dose of VSV into a mouse dramatically augmented the CTL response against the VSV-Np epitope. This response cannot be attributed to an increase in cytokine release, for example a viral-mediated interferon response, because the response of the CTLs produced by co-infection of VV-PJS-5 vector with 3.6×10^4 ($TCID_{50}$) dose of VSV was not augmented (Figure 3A). It has been suggested that cells infected with recombinant VV may be altered their antigen processing, possibly due to an

alteration of their proteasome function [10] . If this was true, the observed augmentation of VSV-specific CTL response by VV-hTAP1,2 infection may also be explained by a combined effect of both the altered proteasome function and the over-expression of TAP. One method to address this possibility is to use recombinant vaccinia virus carrying a VSV-Np 52-59 epitope minigene (VV-VSV-Np) instead of VSV as a source of antigenic peptide. The epitope generated by this virus does not require degradation by the proteasome in the cytosol of infected cells. Therefore any augmentation of the CTL response can be attributed to a single parameter: TAP over-expression. Inoculation of 3×10^6 (pfu) subunits VV-VSV-Np together with 3×10^4 (pfu) VV-hTAP1,2 dramatically enhanced the VSV-Np 52-59 epitope-specific CTL response as shown in figure 3B. We, thus, have confirmed that TAP over-expression alone is sufficient to augment the K^b-restricted VSV epitope-specific CTL response. Furthermore, we confirm that VV infection alone does not enhance expression of proteasome components (data not shown).

It is interesting to study whether the augmentation of the immune response depends on the titer of VV-hTAP1,2 inoculation. Increasing the inoculated dose from 3×10^3 (pfu) to 3×10^4 (pfu) VV-hTAP1,2 together with 3×10^6 (pfu) VV-VSV-Np resulted in an increase of the augmented VSV epitope-specific CTL response. Higher titers of VV-hTAP1,2 still augmented the VSV epitope-specific CTL response, however, at lower levels than the 3×10^4 (pfu) VV-hTAP1,2 inoculum (Figure 4). The explanation for this decrease in the helper effect mediated by VV-hTAP1,2 may be that at high doses of co-inoculated VV-hTAP1,2, peptides derived from VV start to compete with VSV-derived peptides for binding and presentation by MHC Class I of the infected cells. This will change the cell surface K^b-restricted antigenic peptide pool, resulting in a shift of

the dominant immune response from VSV-Np to VV-antigens. In our experiments, the activity of CTL precursors generated by the VV-VSV-Np but not the VSV inoculum requires *in vitro* re-stimulation with VSV-Np peptide (see material and methods), suggesting that the frequency of the VSV-Np epitope specific CTL precursors from the VV-VSV-Np inoculation is less than that in VSV inoculation. This could be explained by the influence of the cell surface VV-antigen concentration on the dominant immune response against VSV-Np epitope. In accordance with this, Levitsky et al. report that the concentration of surface antigenic peptides from two epitopes derived from one viral protein is an important parameter for inducing a dominant immune response [11]. Another possibility is that a competitive mechanism for viral entry may occur during co-infection with two viruses.

To investigate the specificity of this augmentation phenomenon further, we tested whether the specific CTL precursor (CTLp) frequency, TAP expression and the peptide transport capacity in the immunized mice were enhanced. First, a limiting dilution analysis (LDA) was performed to analyze the difference between the CTLp frequency of the mice injected either VV-hTAP1,2 (3×10^4 pfu) + VSV (3.6×10^4 $TCID_{50}$) and VV-PJS-5 (3×10^4 pfu) + VSV (3.6×10^4 $TCID_{50}$). The LDA data indicated that VV-hTAP1,2 + low dose of VSV infection elicited a higher frequency of VSV-Np specific CTL precursors (frequency (f) = 1/6401) compared with VV-PJS-5 + VSV low dose (f=1/41,585) (figure 5). Thus, more than a 6.5 fold increase in the CTLp frequency ($P<0.005$) was observed in the presence of TAP.

The priming of T cells requires cell-to-cell contact by their surface molecules [12]. Therefore, the antigen-presenting cells adjacent to T cell areas play

a critical role. The CTLs used in our experiments are generated in spleens that contain APCs such as B cells, dendritic cells and macrophages. An increase of TAP expression and peptide transport activity in the neighboring APCs, would then explain the high frequency of specific CTLp. In support of this hypothesis, the mouse splenocytes infected with 3 X 10^4 (pfu) VV-hTAP1,2 for one day clearly express the introduced human TAP1 protein (figure 6A), including B cells, macrophage and dendritic cells, respectively (figure 6C). The RT-PCR analysis of human and mouse TAP expression revealed that the splenocytes expressing human TAP may very (figure 6B) and, however, significant transport of an ^{125}I-labeled peptide-library[13] into the lumen of the ER was observed (figure 5D). These data both individually and collectively support the contention that the augmentation we witness is due to increased TAP expression and TAP activity in antigen presenting cells of the VV-hTAP1,2 inoculated spleens.

The peptide transport through TAP is thought to be essential for MHC class I antigen presentation. We have shown that at low dose of VSV inoculation, over-expression of TAP molecules in the APCs can augment VSV-Np epitope specific CTL response. It would be interesting to test if TAP over-expression could augment anti-viral response against other epitopes. Therefore, we used another well-characterized CTL epitope derived from the Sendai virus, Sendai-Np, to generalize the effect of TAP over-expression. The dose of Sendai virus inoculation to achieve a minimal immune response is 1.58 X 10^5 ($CEID_{50}$) units (data not shown). As expected, co-infection of this concentration of Sendai virus, along with 3 X 10^4 (pfu) VV-TAP1,2, significantly augmented a Sendai-Np specific CTL compared to the controls (figure 7).

We have shown here that CTL responses against VSV-Np and Sendai-Np

epitopes are augmented by over-expression of TAP in mice inoculated with VV-hTAP1,2. It is unlikely that this augmentation is due to the more efficient translocation of VSV-derived peptides by human TAP and/or interspecies (human/mouse) TAP heterodimers compared to mouse TAP complexes. It has been reported that human TAP preferentially transports peptides containing hydrophobic or positively charged amino-acids at their C-terminus, while mouse TAP favors peptides with hydrophobic amino-acids at their C-terminus [14]. The VSV-Np and Sendai-Np are two murine K^b-restricted epitopes that contain the same hydrophobic residue (Leucine) at C-terminus. The transport of these peptides by human TAP would compete with an additional peptide pool containing positively charged C-terminal residues. This might lead to a reduced amount of VSV-Np or Sendai-Np entering the ER-lumen for surface presentation through human TAP heterodimers. In addition, the Sendai-Np epitope has an aromatic residue (phenylalanine) at the peptide position 1 (N-terminus) and this has a strong deleterious effect for human TAP binding [15] and for transport. Therefore, we conclude that the transport of Sendai-Np by human TAP would be no better than mouse TAP. For interspecies TAP's, the transport of peptides is restricted to those with hydrophobic C-terminal residues, similar to mouse TAP [16]. This would imply that when mouse TAP1 or TAP2 associates with its human TAP counterpart, they play a dominant role in selecting the peptides for transport. Furthermore, once the transport-permissive peptide, for example Sendai-Np, binds to interspecies TAP's, the phenylalanine residue at N-terminus contacting to human TAP counterpart may limit its binding capacity and therfore limit its transport. For these reasons, we conclude that the augmentation of CTL response against viruses in our experiments is justified by TAP over-expression rather than the increased efficiencies of interspecies TAP heterodimers.

It has been suggested that in the absence of viral infection only one-third of all TAP molecules actively translocate peptides while during an acute viral infection active TAP molecules are increased significantly and this is owing to rapidly increase the intracellular peptide pool available to TAP [17]. Our results (figure 3A, 3B and 7) indicate that, although viral infection induces more active endogenous TAP, this level of the active TAP is not sufficient to augment immune response against specific viral epitopes that we investigated (as seen in our controls) without the addition of exogenous TAP. This is likely due to the competition in transport of the small-epitope peptide pool with self- and other viral peptides, which limits the sufficient amount of the epitopes reaching to the cell surface. Thereby an additional TAP supply is required to increase absolute amounts of active TAP (but may or may not change active/inactive TAP ratio) and thus TAP over-expression can efficiently transport the epitopes, resulting in the augmentation of the immune response.

With the use of adjuvant, the immune response can be modulated for a MHC class I or II response. Adjuvant like immunostimulating complexes (ISCOMs), that are made of non-covalenty bound complexes of Quil A, cholesterol, and amphipathic antigen can stimulate a $CD8^+$ CTL response [18]. Similarly, the T cell costimulatory molecule B7 has been shown to enhance protection against poorly immunogenic tumours [19,20]. In addition a wide variety of cytokines have been used to direct responses to either a CTL mechanism or T helper response. For example, interleukin-2 (IL-2) and IL-12 have been used to elicit a Th1 response that is more conducive to cytotoxic mechanisms [21-25]. One adjuvant that has been widely used in animals is Freund's complete adjuvant (FCA) that is an emulsion containing heat killed *mycobaterium tuberculosis*. Despite the strong antibody responses that FCA

produces, it is too toxic to be used in humans. However, derivatives for the minimal structure of the mycobaterium in FCA that is needed for adjuvanticity, N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP), such as murabutide do not have as many toxicity problems [26].

We have demonstrated that augmentation of TAP expression in mice does increase the immune response to the VSV and Sendai. This strategy does not increase autoimmunity while it boosts specific viral immunity significantly, because no killing of uninfected syngeneic targets was observed (data not shown). These data surprisingly imply that TAP expression or activity is limiting for viral responses in normal cells and is the first component of the antigen processing pathway demonstrated to be in short supply in healthy mice. Thus, TAPs may act as an adjuvant in healthy or immunocompromised individuals. When designing subunit vaccines, one may want to reduce the amount of inoculum used for vaccination. This is important not only for efficiency in distributing large amounts of vaccine [27] but is also important when larger doses of a weakly immunogenic antigen are required. The inclusion of TAP in vaccination regiments could address these problems. An additional advantage of including TAP as an adjuvant is its ability to increase peptide transport of a number of immunogenic peptides simultaneously. In a virus containing a complex array of peptides, one could envisage that the inclusion of TAP would increase the delivery of most epitopes. Furthermore, this would aid in the delivery of diverse peptide for binding to most HLA alleles expressed in the population being immunized. TAP could be used as an adjuvant in peptide vaccines but it does not have to be restricted to viral vectors. For example, it could also be injected in other forms such as in DNA plasmids attached to gold particles or any other system that inserts the TAP complex directly into the cell's

protein processing pathway [28]. Finally, the use of TAPs as an adjuvant has the advantage that we have a solid intellectual understanding of its mechanism of action. This appears to be lacking in the case of many other generalized adjuvants [29]. Future clinical experiments will help to further establish whether the inclusion of TAP in vaccine regiments has advantages over existing protocols, whether other components of the intracellular antigen processing pathway(s) are also limiting in healthy individuals.

Materials and Methods

Animals

The mouse strains C57BL/6 (H-2^b) were obtained from Jackson Laboratories and housed and bred by Willem Schoorl at the Biotechnology Breeding Facility (University of B.C.). The mice were maintained according to the guidelines of the Canadian Council on Animal Care. The mice used in the experiments were between 6 and 12 weeks of age and were sacrificed by CO_2 asphyxiation.

Experimental protocol for augmentation of T cell-mediated immune response.

H-$2K^b$-restricted VSV- or Sendai virus-specific CTLs were generated by i.p. injection of either VSV or Sendai virus alone or one of these viruses plus recombinant vaccinia virus (VV) carrying with (VV-hTAP1,2) or without (VV-PJS-5) human TAP 1 and TAP 2 genes into C57BL/6 mice with the viral dose indicated in each figure. The viral dose used as following: plaque forming units (pfu) for vaccinia virus, tussue culture infecton dose ($TCID_{50}$) units for VSV and cheken embryo infection dose ($CEID_{50}$) units for Sendai virus. After 5-6 days immunization, the splenocytes were removed and cultured in RPMI-1640 complete medium containing 10% heat-inactivated HyClone FBS (GIBCO BRL), L-glutamine, 100IU/ml penicillin, 100μg/ml streptomycin, Hepes, 0.1 mM non-essential amino acids, 1 mM Na-pyruvate, and 50 μM 2-ME. The splenocyte cultures were incubated at 3 X 10^6 cells/ml at 37°C for 3 days with the peptide (1 μM Sendai-Np 324-332 peptide, FAPGNYPAL) for Sendai-specific effectors and (1 μM VSV-Np 52-59 peptide, RGYVYQGL) for the effectors generated by VV-VSV-Np inoculation or without a peptide for the effectors generated by VSV inoculation. The erythrocytes were removed from the splenocytes before 3 days culture (for VSV-specific effectors) or after (for Sendai-specific effectors).

The cytotoxic activity was measured in standard 4-h 51Cr release assays. The RMA target cells were pulsed with 5-25 μM either VSV-Np peptide 52-59 (RGYVYQGL) or Sendi-Np peptide 324-332 (FAPGNYPAL) for relevant CTL response. The targets were labeled with Na51CrO4 (70 $\mu Ci/10^6$ cells) for 1 hr at 37°C and cytotoxic activity was assayed in a standard 4 h 51Cr release assay. The cytotoxicity tests were done in 96 V-shaped well plates at many effector:target ratios.

Analysis of Frequency of VSV-specific CTL Precursor.

The frequency of CTL precursor was detected by limiting dilution analysis (LDA). Briefly, the assay was performed in U-bottom 96-well plate supplied with the complete medium containing 20U/ml recombinant mouse IL-2, 5% supernatant of con A-stimulated rat splenocytes and 0.1 M α-methyl-D-mannoside. Wells contained graded concentrations of the immunized splenocytes and both irradiated cells, 1×10^5 syngenetic splenocytes as the feeders and 3×10^3 VSV-Np 52-59 peptide-pulsed RMA cells as the stimulators. The cells were cultured at 37°C for 7 days and at day 6, 80 ml cultured medium in each well was replaced with same amount of fresh one. On day 7, a standard CTL assay was performed by replacing 100 ml supernatant with 5 mM VSV-Np peptide-pulsed, ^{51}Cr-labeled RMA cells in each well as targets. Kinetic analysis and CTL precursor (CTLp) frequency determinations were performed by the statistical methods of X^2 minimization as described by Taswell [30].

Detection of TAP Expression and Activities.

Human TAP1 expression in immunized mouse splenocytes were determined by immunobloting. Total extracts from 1×10^6 cells were separated on 10% polyacrylamide-SDS gels and blotted onto nitrocellulose filters. The blots

were probed with TAP C-terminus-specific rabbit antiserum (gifts from Dr. Monaco, J. J.) at a 1:1000 dilution for anti-human TAP1. The blots were then incubated with horseradish peroxidase-labelled anti-rabbit antibodies at a 1:10,000 dilution. The immunocomplexes were visualized by enhanced chemiluminescence (ECL) according to the instructions of the manufacturer (Amersham, UK). The naive mouse splenocytes were used as negative controls.

Confocal fluorescence microscopy analysis of human TAP expression in antigen presentation cells was performed with a confocal system. Briefly, C57/B6 mice were inoculated with 3×10^4 (pfu) VV-hTAP1,2 or VV-PJS-5 vector. After one-day inoculation, the splenocytes were prepared by depletion of the erythrocytes with a buffer consisting of NH_4Cl 8.29 g, $KHCO_3$ 1.0 g, EDTA 0.0372 g per 1 liter.destilled water (pH 7.4). 5×10^6 splenocytes were then allotted into V-bottom 96 well plate and washed twice in PBS before fixing with 2 % (w/v) paraformaldehyde in PBS for 15 minutes and permeabilizing with 0.1 % (w/v) saponin in 2 % (w/v) BSA/ PBS for 15 minutes at room temperature (this treatment lose the specific growth pattern of DC cells). Afterwards, the splenocytes were blocked with 2 % (w/v) BSA/ PBS for 1 hour at room temperature for antibody staining. To detect human TAP1 expression in B cells, macrophages and DCs, the splenocytes labeled with rabbit anti human TAP1 serum at 1:1000 dilution (Stressgen Biotechnologies Corp) were co-stained with either rat anti mouse B220 (1:100), rat anti mouse MAC-1 (1:150) or rat anti mouse NLDC-145 (from ATCC) (1:100) antibodies for 30 minutes at room temperature. Excess antibodies were extensively washed before staining the cells with fluorochrome conjugated secondary antibodies (Alexa 488 conjugated goat anti rabbit IgG and Alexa 568 conjugated goat anti rat IgG). 1:1000 diluted secondary antibodies were used to stain the cells at room temperature for 30

minutes, followed by extensively washing with 0.1% (w/v) saponin, 2% (w/v) BSA/ PBS. The coverslip was mounted with 5 µl of Slow Fade (Molecular Probes, Eugene, OR) before sealing it onto a glass slide with nail polish. The confocal micrographs were collected using the BioRad Radiance and images were processed using NIH imaging system.

RT-PCR analysis of human and mouse TAP mRNA expression was performed in VV-PJS-5 or VV-hTAP1,2 inoculated mice splenocytes. Total RNA was extracted from the 20mg of splenic tissue of the viral infected mice using the RNeasy Kit (Qiagen) according to manufacturer's protocol. Random-primed cDNA was generated using the RETROscript, RT-PCR Kit (Ambion) following the manufacturer's instructions. We then used 0.5mg of cDNA from each spleen to amplify sequences corresponding to human TAP1 and TAP2 and mouse TAP1 and TAP2 using the following primer sets: *GGGGACAGCTGCTGTTGGAT* and *AGTACACACGGTTTCCGGATCAAT* corresponding to the 1709-1728 and 2024-2001 of human TAP1, respectively; *GGACAGGTGCTGCTGGATGA* and *TCGCACTGCACATCTAGGGC* corresponding to 1600-1619 and 1952-1933 of human TAP2, respectively; *GCTGTGGGGACTGCTAAAAG* and *TATTGGCATTGAAAGGGAGC* corresponding to 144-163 and 808-789 of mouse TAP1, respectively; and *GACTTGCCTTGTTCCGAGAG* and *CTGTGCTGGCTATGGTGAGA* corresponding to 254-273 and 646-627 of mouse TAP2, respectively. The S15 ribosomal subunit primer set was obtained from Ambion. For all targets, the PCR reaction consisted of 30 cycles of amplification at an annealing temperature of 56°C using Platinum Taq polymerase (Invitrogen), according to manufacturer's instructions. One tenth of the product of each PCR reaction was examined by agarose gel electrophoresis. We measured the intensity of the 361bp RT-PCR product of the S15 ribosomal

subunit generated from various spleens in order to ensure that the reaction kinetics and starting material of cDNA in each reaction was equivalent.

TAP heterodimer activities were detected by streptolysin-O mediated peptide transport assays as described by Androlewicz et al. [31] with minor modifications. Briefly, a peptide-library [13] which contains 3240 different peptides with a glycosylation site (NXT) in each was labeled with 125I by chloramine T-catalyzed iodination to a specific activity of 10 Ci/mmol. 2 X 10^6 splenocytes from naive, TAP-/-, VV-PJS-5- or VV-hTAP1,2-immunized mice were permeabilized with 2 IU/ml streptolysin-O (Murex, Norcross, GA) for 15 min at 4°C. After removing unbound streptolysin-O and the cells were resuspended in 37°C intracellular transport buffer (50 mM Hepes, pH 7.0, 78 mM KCl, 4 mM MgCl2, 8.37 mM CaCl2, 1 mM EGTA, 1 mM dithiothreitol (DTT). Adjust pH to 7.3 with KOH) for 5 min to initiate pore formation. The iodinated peptide-library (ref) (~66 ng) was then added immediately. The incubation was continued for another 10 min in the presence or absence of 10 mM ATP (Sigma Chemical Co., St. Louis, MO). Afterwards, the cells were transferred to ice and were lysed in a buffer containing 1% NP40, 150 mM NaCl, 5 mM MgCl2, 50 mM Tris-HCl pH 7.5. The nuclei were removed by centrifugation of samples at 14,000 rpm for 10 min. Translocated peptides that had been glycosylated in the ER were recovered by absorption to concanavalin A-Sepharose beads (Pharmacia Diagnostics, AB). The beads were washed five times in lysis buffer. The associated radioactivity was measured in a g-counter (model 1282CS; LKB Pharmacia).

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Figure Legend

Figure 1. Determination of VV-hTAP1,2 function

A CTL assay was performed to determine the ability of VV-hTAP1,2 to produce a functional TAP that transports the peptide. The targets of T2/K^b were infected (MOI=10) for 6 hours with either vaccinia carrying VSV-Np 52-59 minigene (VV-NP), VV-NP with the VV-hTAP1,2 or VV-NP with vaccinia vector alone (VV-PJS-5). The presentation of peptide was determined indirectly by target cell lysis by VSV specific CTL in a 4-hr ^{51}Cr release assay.

Figure 2. The VSV-specific primary CTL generation in VSV-infected mice is viral dose-dependent. The immunized splenocytes derived from the mice injected with the indicated VSV $TCID_{50}$ doses were tested for their cytotoxic activity by using VSV-Np 52-59 peptide-pulsed RMA cells as target. A standard 4-hr ^{51}Cr release assay was performed.

Figure 3. TAP heterodimer enhances the VSV-Np epitope-specific primary CTL responses. The immunized splenocytes derived from the mice injected with either VSV (Fig. 3A and B) or VV-VSV-Np (Fig. 3C) with VV-containing with or without human TAP1,2 genes were tested for their cytotoxic activity by using VSV-Np 52-59 peptide-pulsed RMA cells as target. A standard 4-hr ^{51}Cr release assay was performed. Each legend, indicated a concentration of viral injection, is shown as following: A. VV-PJS-5 + VSV low (3×10^4 pfu + 3.6×10^4 $TCID_{50}$), VV-TAP1,2 + VSV low (3×10^4 pfu + 3.6×10^4 $TCID_{50}$), VSV low (3.6×10^4 $TCID_{50}$) or VSV high (1.5×10^7 $TCID_{50}$); B. VV-PJS-5 + VV-VSV-Np (3×10^4 pfu + 3×10^6 pfu), VV-hTAP1,2 + VV-VSV-Np (3×10^4 pfu + 3×10^6 pfu), VV-VSV-Np (3×10^6 pfu) and VSV high (1.5×10^7 $TCID_{50}$).

Figure 4. Augmentation of VSV-Np epitope specific CTL response requires suitable amount of VV-hTAP1,2 inoculation. The immunized splenocytes derived from the mice injected with 3 X 10^6 (pfu) VV-VSV-Np together with either VV-PJS-5 or VV-hTAP1,2 within the concentration indicated in this figure were tested for their cytotoxic activity by using VSV-Np 52-59 peptide-pulsed RMA cells as target. A standard 4-hr ^{51}Cr release assay was performed.

Figure 5. VSV-Np epitope specific CTLp frequency is greatly enhanced by introducing TAP 1 and TAP 2 genes into VSV-immunized mouse. The immunized splenocytes from the mice injected with either VV-TAP1,2 plus VSV low or VV-PJS-5 plus VSV low with viral doses indicated in the legend of figure 2 were analyzed for VSV-Np epitope specific CTLp frequency. A limiting dilution analysis was performed by using VSV-Np peptide-pulsed RMA cells as target.

Figure 6. Mice splenocytes express introduced human TAP and efficiently transport a peptide-library. 3 X 10^4 (pfu) of either VV-PJS-5 or VV-TAP1,2 were injected i.p. into mice. After one day's injection, the splenocytes were removed and divided into four aliquots for detecting human TAP1 expression and TAP transport activities. The naive (Normal) and TAP-/- mice splenocytes are used as controls. A) The immunobloting analysis was performed to detect human TAP 1 expression. B) The RT-PCR analysis was performed to detect human and mouse TAP1 and TAP2 expression. C) The confocal fluorescence microscopy analysis was carried out to identify human TAP1 expression in B cell, Macrophage and DC. The red or green colors indicate the cell surface markers or human TAP1 protein, respectively. I and II ---- B cells from the immunized mouse spleens infected with either VV-PJS-5 (I) or VV-hTAP1,2 respectively (II). III and IV ---- Macrophage and DC cells from VV-hTAP1,2 inoculated mouse spleen. The

analysis reveals that 51% of nucleated splenocytes clearly express human TAP1 protein. D) TAP function was tested by a peptide transport assay using an ^{125}I-labeled peptide library as the reporter.

Figure 7. TAP heterodimer enhances the Sendai virus-specific primary CTL response. The immunized splenocytes derived from the mice injected either Sendai virus alone or Sendai virus plus VV carrying with or without human TAP 1 and TAP 2 genes were tested for their cytotoxic activity by using 5mM Sendai-Np (324-332) peptide-pulsed RMA cells as target. A standard 4-h ^{51}Cr release assay was performed. Each legend is shown as following: VV-PJS-5 + Sendai low----3 X 10^4 (pfu) VV alone plus 1.58 X 10^5 ($CEID_{50}$) VSV, VV-TAP1,2 + VSV low----3 X 10^4 (pfu) VV-TAP1,2 plus 1.58 X 10^5 ($CEID_{50}$) Sendai virus, Sendai low-----1.58 X 10^5 ($CEID_{50}$) Sendai virus or Sendai high-----1.58 X 10^7 ($CEID_{50}$) Sendai virus.

Figure 1

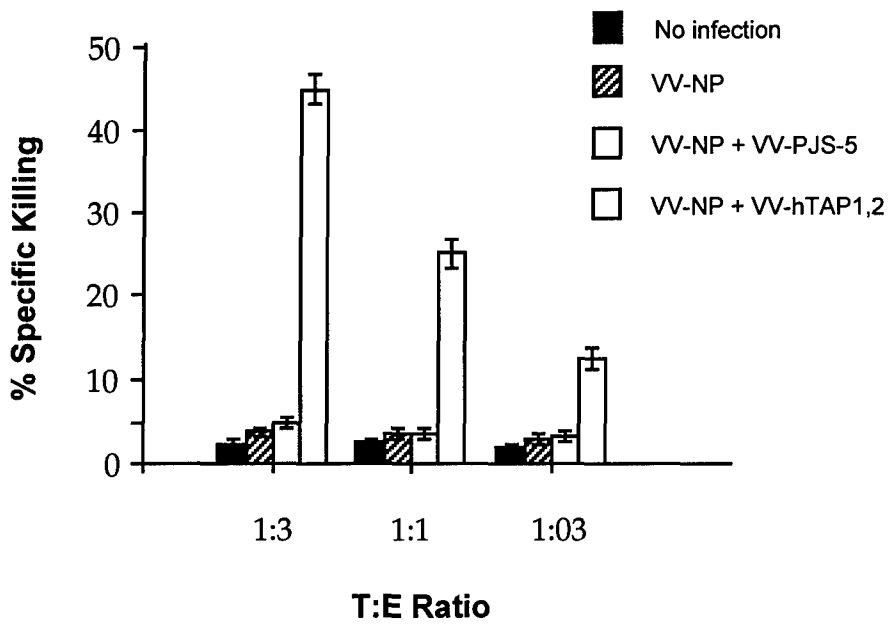

No infection
VV-NP
VV-NP + VV-PJS-5
VV-NP + VV-hTAP1,2
% Specific Killing
50
40
30
20
10
0
1:3
1:1
1:03
T:E Ratio

Figure 2.

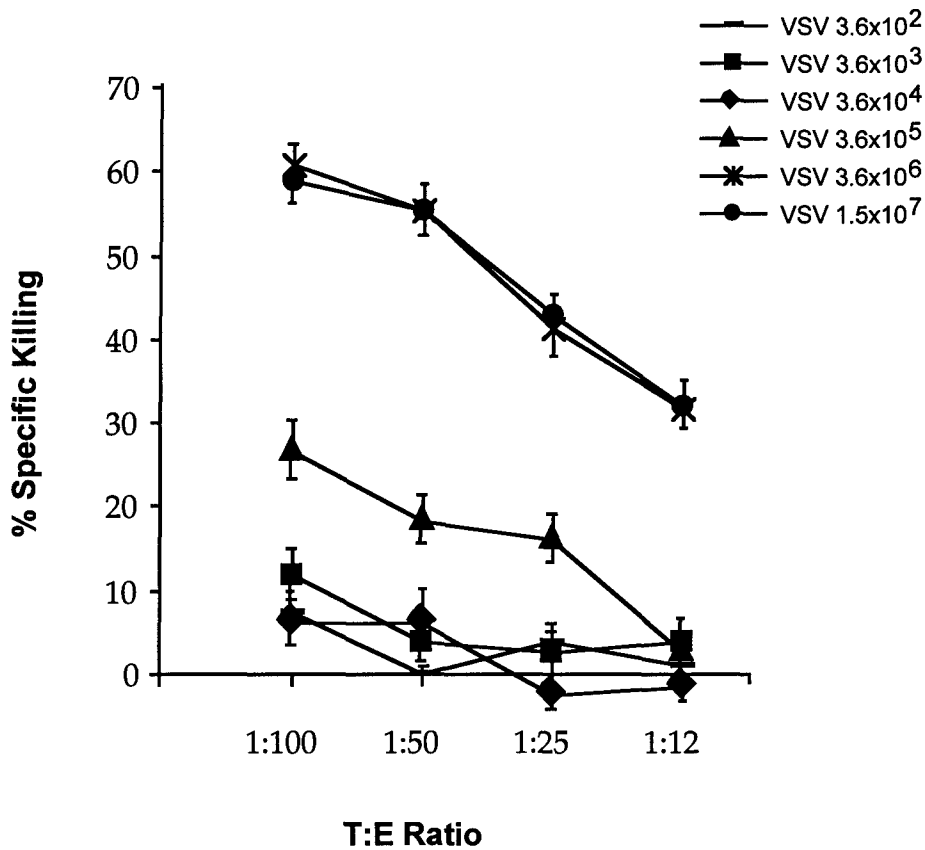

VSV 3.6x10^2
VSV 3.6x10^3
VSV 3.6x10^4
VSV 3.6x10^5
VSV 3.6x10^6
VSV 1.5x10^7
70
60
50
40
30
20
10
0
% Specific Killing
1:100
1:50
1:25
1:12
T:E Ratio

Figure 3.

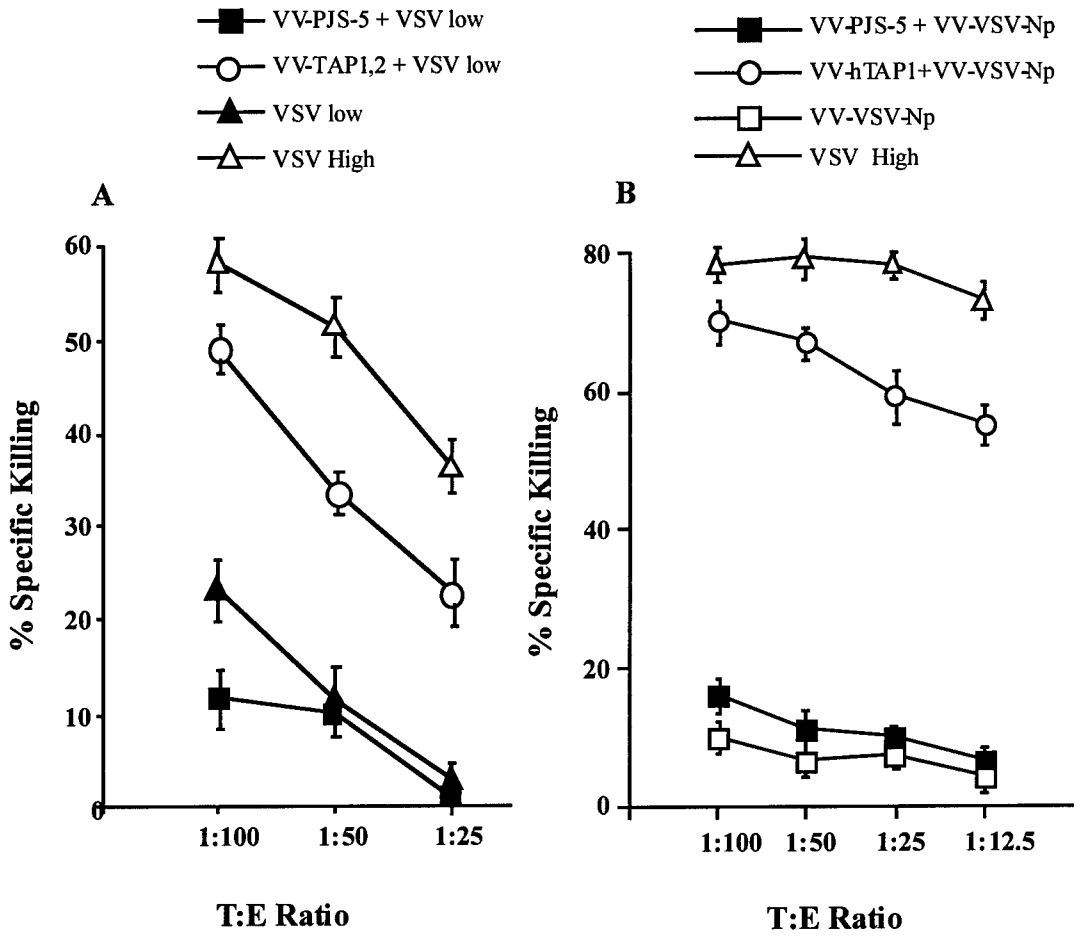
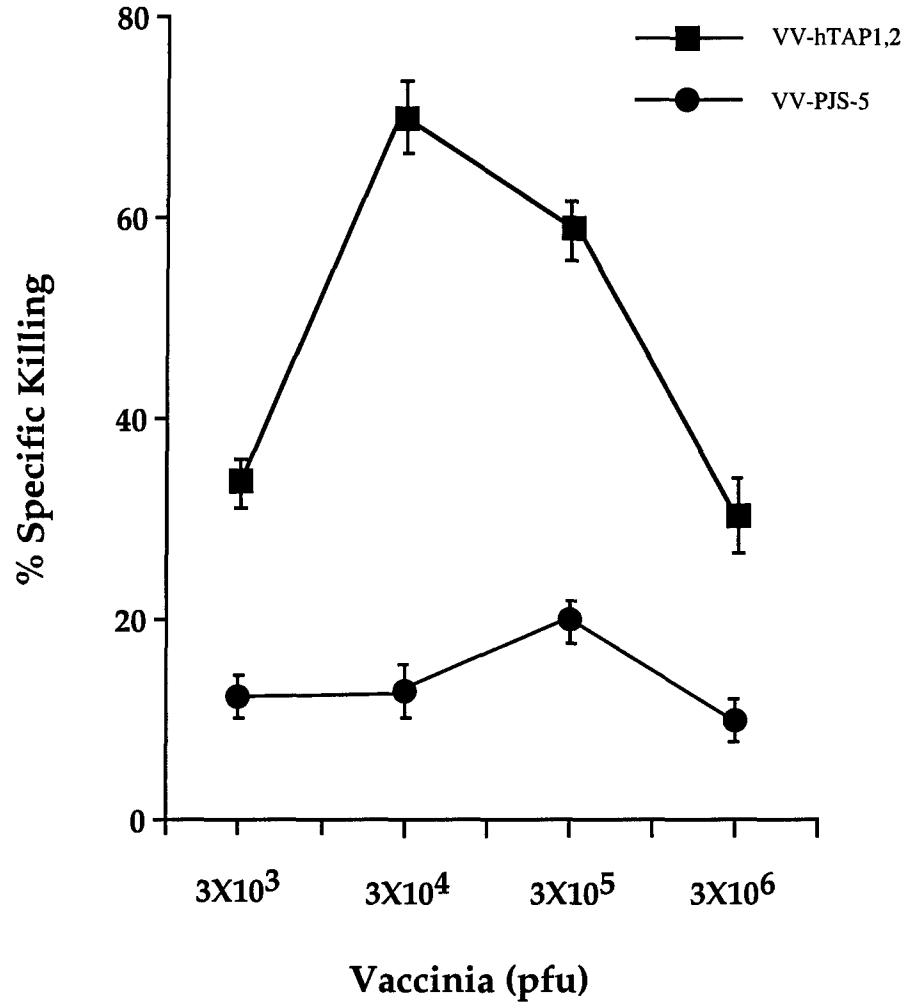

VV-PJS-5 + VSV low
VV-TAP1,2 + VSV low
VSV low
VSV High
A
% Specific Killing
60
50
40
30
20
10
0
1:100
1:50
1:25
T:E Ratio
VV-PJS-5 + VV-VSV-Np
VV-hTAP1+VV-VSV-Np
VV-VSV-Np
VSV High
B
% Specific Killing
80
60
40
20
0
1:100
1:50
1:25
1:12.5
T:E Ratio

Figure 4


80
60
40
20
0
% Specific Killing
VV-hTAP1,2
VV-PJS-5
3X10^3
3X10^4
3X10^5
3X10^6
Vaccinia (pfu)

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